Volume 24, No. 2

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БИОХИМИЯ

BIOKHIMIYA

translated from Russian

CONSULTANTS BUREAU, INC.

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THE MECHANISM OF UPTAKE OF LABELED AMINO ACIDS BY PROTEINS

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In previous work [1, 2] we showed that when fertilized and unfertilized hens' eggs were incubated with radioactive amino acids, no radioactivity was found in the proteins of either the yolk or the white, while the proteins of the developing embryo had a high radioactivity. These results allowed us to conclude that the uptake of amino acids in the protein molecules occurred only at the moment of their new formation and that there evidently was not any process in the organism for the independent uptake of amino acids at different times in the already formed protein molecule. In the present work this observation has again been confirmed.

EXPERIMENTAL

The experiments were carried out on rats in which liver function had been excluded. We used rats weighing 300-350 g for the experiment. Under general ether anesthesia, the gastrointestinal tract was fully removed from the experimental animals and the vessels which supplied the liver were ligated.*

In order to avoid the development of hypoglycemia in the "operated" rats we injected subcutaneously, every half hour from the end of the operation, a 20% solution of glucose which contained 22 units of insulin per 100 ml of solution, injecting 0.5 ml per 100 g of body weight. Since the eviscerated animals could not maintain a constant body temperature, they were kept until the end of the experiment at 27°, which according to the literature [3], is best for the survival of rats. As controls we used animals which had undergone the operation of laparotomy.

Immediately after operation the control and operated animals were injected with a solution of radioactive methionine at 3000 counts/ min per g weight. The weight of the operated animals was taken as the difference between the weight before the operation and the weight of the viscera removed.

After the end of the experiment blood was taken from the heart and centrifuged; from its serum we isolated the albumin by electrophoresis on moist starch blocks [4]. The electrophoresis lasted 9-10 hours (voltage 300-350 v, current strength 20-25 ma). At the end of the electrophoresis the starch block was cut into sections. The portions of the block which contained the albumin fraction were transferred to a test tube, twice washed with small portions of cold distilled water, and 0.02 ml of the eluate was taken for a test of the purity of the albumin (by paper electrophoresis); the albumin was precipitated from the rest of the eluate by a 5% solution of trichloroacetic acid (TCA). The protein was washed three times in the centrifuge with the 5% TCA solution and then with a 1:1 mixture of alcohol and ether, then finally with ether. A target was prepared from the dry protein powder for determining the activity.

The activity of the protein was determined in a counter with a mica window 3.9 mg/ cm^2.

As Table 1 shows in rats whose liver function was excluded by the operation there was no uptake of radioactive methionine in the albumin of the blood; in animals with normal livers this process goes on very intensely. These results indicate that radioactive amino acids can be included in the albumin molecule only when it is newly formed (which occurs in the liver) and there is evidently no independent process of "renewal" which is characterized by independent uptake and removal of amino acids at different times from the already-formed albumin molecule. The question still remains unsettled, however, as to whether there is a process of "renewal" of the protein at the time of its synthesis. It seems to us that one of the ways to decide this question is to study the interdependence between the uptake of individual amino acids at the time of synthesis of some individual protein.

* The operation of evisceration was carried out with the advice and direct participation of Prof. N. A. Yudaev, to whom we express our thanks.

Therefore the further development of this work is our investigation of the relation between the separate amino acids during their uptake in the proteins of the developing chick embryo.

TABLE 1. Uptake of Methionine-S^{35} in the Blood Albumin of Sham-Operated and Experimental Rats.

Time after adding methionine-S^{35}, hours	Activity of albumin, counts/min per 10 mg protein	
	Experimental rats	Sham-operated rats
4	0.2	32
6	0	–
8	0	–

As the subject of study we chose the proteins of the chick embryo in the early stages of development, when the processes of protein synthesis prevail. We tried to determine the rate of renewal in the developing embryo protein at different time intervals of the two sulfur-containing amino acids, methionine and cystine. For this purpose we injected into the egg at 5-7 days incubation radiomethionine calculated at 100 counts/min per g weight of egg. The injection into the egg of one methionine is equivalent to the simultaneous injection of two amino acids, methionine and cystine, because of the easy transformation of part of the added methionine-S^{35} into cystine-S^{35}. At different time intervals we removed the embryos from some of the eggs and washed them several times with distilled water to free them from possible radioactive contaminants of the surrounding medium; we removed the muscle and internal organs, which were combined and after homogenization the protein was separated by precipitation with TCA. Part of the protein was used for determining the total sulfur according to Pirie [11]. The cystine-cysteine sulfur was separated from the rest of the protein after its oxidation with alkaline permanganate according to Callan and Toennies [12]. On the basis of the determination of the activity in $BaS^{35}O_4$ we have calculated the specific activities of cystine and methionine.

The results are summarized in Table 2.

TABLE 2

Change with Time in Specific Activity of Cystine and Methionine in the Proteins of the Chick Embryo

Experiment No.	Age of embryo (days)	Time after injection of methionine-S^{35} into embryo (hours)	Specific activity		a/b
			Methionine, a	Cystine, b	
Experiment 1	6	4	1.88	0.16	11.8
	5	24	5.75	0.62	9.3
	6	48	9.48	0.72	12.1
	7	96	4.55	0.39	11.7
Experiment 2	6	48	8.00	0.91	8.8
	7	72	5.28	0.61	8.7
	8	93	6.09	0.50	12.7

The results in Table 2 show that the ratio of specific activities of methionine and cystine in the embryo proteins measured at different time intervals after one injection of methionine has a nearly constant value.

This constant ratio in rate of uptake of the two amino acids in the protein permits the assumption that the protein of the chick embryo is synthesized directly from the amino acids and that at the time of the synthesis there is no "renewal" of the tissue protein of the chick embryo.

RESULTS

In the first part of our study we have succeeded in showing that in rats submitted to an operation for functional exclusion of the liver, radioactive methionine is not included in the composition of the molecule of blood albumin. As is known, the synthesis of blood albumin occurs in the liver [5-9]. The absence of labeling in rat blood albumin when the liver is removed shows most convincingly that the radioactive amino acids are taken up by the albumin only at the time of its synthesis, and after the synthesis the protein molecule does not undergo continuous renewal by constant uptake and loss of the amino acids which compose it. Analogous results were obtained

by Konikova and Uchitel' [10] who succeeded in showing with the aid of glycine-C^{14} and methionine-S^{35} that the inclusion of amino acids in the composition of antibodies occurs only at the time of their formation and that "passive" antibodies, antibodies in vitro, and antibodies circulating in the blood of an organism cannot be renewed.

If we can show that the uptake of amino acids occurs only at the moment of synthesis of the protein molecule and there is no process of renewal of the separate amino acids in the protein as an independent process, separate from the new formation, then the question still remains of the possibility of "renewal" at the time of synthesis of the protein molecule. In fact, it may be that the process of biosynthesis of proteins includes as separate elements of the reaction, both new formation of proteins and the renewal of their constituent parts, which usually do not occur as separate processes in the organism. One of the possible ways to solve this question is to study the interrelation between the uptake of separate amino acids during the synthesis of some individual protein. If we assume that synthesis of the protein molecule goes from the free amino acids of the metabolic pool, then in the new formation of proteins there should be a quantitative relation between the rate of utilization of the different amino acids which are used for construction of the protein. If renewal processes occur in the new formation of proteins, then it is not very probable that we will have simultaneous uptake of amino acids and the same rate of circulation of all the amino acids in the protein molecule. Our observed constancy of ratio of rate of uptake of two amino acids in the protein permits us to assume that the protein of the chick embryo is synthesized from amino acids and that at the time of synthesis there is no "renewal." The same results were obtained by Muir [13] on globin, by Askonas, Campbell, and Work [14,15] on milk proteins, and by Simpson and Velick [16, 17] on aldolase and glycerol dehydrogenase.

SUMMARY

In rats submitted to an operation to exclude liver function, radioactive methionine is not included in the composition of the blood albumin molecule. The incorporation of amino acids in the serum albumin occurs only at the time of its formation.

The ratio of specific activity of cystine and methionine in the protein of the chick embryo, measured at different time intervals after a single injection of methionine, is nearly a constant value. These results indicate that the protein of the chick embryo is synthesized directly from amino acids and at the time of its new formation there is no renewal of separate amino acids.

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A POLAROGRAPHIC METHOD FOR THE DETERMINATION OF ADRENALINE, NORADRENALINE AND SUBSTANCES WITH SOME PROPERTIES OF ADRENALINE OXIDATION PRODUCTS

A. F. Maslova

The study of the hormones of the adrenal medulla, which exert an important regulatory influence on the general reactions of the organism, is complicated by the fact that until recently there has been no simple, rapid, specific, and highly sensitive method for their quantitative determination.

A number of methods are described in the literature for determining adrenaline and noradrenaline. One of these is the fluorescent method worked out in the laboratory of Utevskii, Butom, Frenkel' and Barts [1]. Osinskaya [2] in the same laboratory worked out a more sensitive fluorescence-analytical method for determining adrenaline (AD), noradrenaline (NAD) and products of their oxidation. By suitable treatment the author found conditions for differentiation of adrenaline and noradrenaline from those products which would change the determination. However, the sensitivity of this method ($0.25 \cdot 10^{-6}$ M) was still insufficient for the determination of the amounts of AD and NAD in the tissues and fluids of the animal organism.

TABLE 1

Amount of Reagents Required for Oxidation of AD or NAD at Different Ranges of Concentration

AD or NAD in mmole/liter · $\cdot 10^{-5}$	KIO_3, normality · $\cdot 10^{-4}$	KI in % · $\cdot 10^{-4}$	H_2SO_4, normality · $\cdot 10^{-4}$	$Na_2S_2O_3$, normality · $\cdot 10^{-4}$
1	1	1	1	1
10	10	10	10	10
100	100	100	100	100

Henderson and Freedberg [3] made a number of polarographic determinations of high concentrations of the crystalline forms of AD and NAD in concentrations of $1 \cdot 10^{-3} - 1 \cdot 10^{-2}$ M.

We have modified the method of these authors for the simultaneous determination of AD and NAD in biological liquids. For the determination we used blood and humor of the eye. This modification raised considerably the sensitivity of the method (to $1 \cdot 10^{-5}$ mmole/liter), its specificity and the speed with which it could be carried out (not more than 15 min is required for determination of AD and NAD).

For the differentiation of AD and NAD we have used the different conditions of oxidizability of these compounds.

Oxidation of AD was carried out with potassium iodate; NAD was oxidized with potassium iodate after addition of sodium acetate to the sample.

Increase in specificity of the method was obtained by polarography of two fractions of the substance which remained after precipitation of the protein in the filtrate under study: substances of nonadrenaline character capable of being polarographed, and in distinction from AD, NAD, stable to formaldehyde (the so-called residual current), and substances which gave a polarographic wave in the presence of formaldehyde. Similar properties

are possessed by oxidation products of adrenaline-like substances with closed side chains which have not yet been identified and are called, in the terminology of Utevskii, "oxidation products" (OP).

In this work we used crystalline AD the base (from the firm "Bayer") and NAD (base) from which we prepared the standard solutions in 2% acetic acid with addition of 0.2 ml of 0.1 N H_2SO_4 and one to two drops of methyl alcohol for better solubility. The standard solutions contained 10 mg of AD or NAD per 1 ml.

TABLE 2

Diffusion Current Constant (K) for Different AD Concentrations in Acetate Buffer at pH 4.2 and t 20°

C, mmole/liter · 10^{-5}	Id, mA		K, Id/C	t
	Found · 10^{-4}	Corrected · 10^{-4}		
0	131,94	—	—	2,74
1	146,60	14,66	14,66	2,69
2	161,26	29,32	14,66	2,80
3	175,92	43,98	14,66	2,74
4	190,58	58,64	14,66	2,76
5	205,24	73,30	14,66	2,90
6	219,90	87,96	14,66	2,94
7	224,56	102,62	14,66	2,95
8	249,88	117,28	14,66	2,68
9	263,88	131,94	14,66	2,91
	$\Sigma\Delta\ K/n = 0$			
0	79,20	—	—	2,76
10	88,00	8,8	8,8	2,74
20	96,80	17,6	8,8	2,75
30	105,60	26,4	8,8	2,80
40	114,40	5,2	8,8	2,74
50	123,20	44,0	8,8	2,80
60	132,00	52,8	8,8	2,76
70	140,80	61,6	8,8	2,84
80	144,60	70,4	8,8	2,83
90	153,40	79,2	8,8	—
	$\Sigma\Delta\ K/n = 0$			
0	52,52	—	—	2,74
100	62,80	6,28	0,628	2,70
200	69,08	12,56	0,628	2,71
300	75,36	18,84	0,628	2,80
400	81,64	25,12	0,628	2,81
500	87,92	31,40	0,628	2,74
600	94,20	37,688	0,628	2,75
700	100,48	43,96	0,628	2,74
800	106,76	50,24	0,628	2,76
900	113,04	56,52	0,628	2,80
	$\Sigma\Delta\ K/n = 0$			

TABLE 3

Change in Height of Wave (H) depending on Concentration of AD and NAD

Temperature	C, AD or NAD mmole per liter · 10^{-5}	Height of wave, H, mm	$E_{1/2}$, $V \cdot 10^{-1}$	Shunt
20,00	30	13	2,10	1:5
20,10	30	13	2,13	1:5
20,20	30	13	2,16	1:5
20,10	90	19	2,10	1:5
20,00	90	19	2,10	1:5
20,00	90	19	2,13	1:3
20,30	1	11	2,16	1:3
20,10	1	11	2,19	1:3
20,00	2	12	2,10	1:3
20,10	2	12	2,10	1:3
20,00	3	13	2,10	1:3
20,00	3	13	2,10	1:3
20,00	9	19	2,10	1:3
20,00	9	19	2,13	1:7
20,10	100	11	3,20	1:7
20,00	100	11	3,00	1:7
20,00	100	11	3,34	1:7
20,00	200	12	3,00	1:7
20,10	200	12	3,00	1:7
20,20	200	12	3,00	1:7
20,00	300	13	3,00	1:7
20,00	300	13	3,00	1:7
20,00	300	13	3,00	1:7
20,00	800	18	3,60	1:7
20,20	800	18	3,30	1:7
20,10	800	18	3,00	1:7

* $E_{\frac{1}{2}}$ — half-wave potential

For determination of AD and NAD over a wide range of concentrations (from 1.10^{-5} to 1.10^{-3} mmole/liter) they were divided into the following zones of concentration:

1) from 1.10^{-5} to 9.10^{-5} mmoles/liter;

2) from 1.10^{-4} to 9.10^{-4} mmole/liter;

3) from 1.10^{-3} to 9.10^{-3} mmole/liter.

For the selection of the concentration of the oxidizing agent, we used the directions of Henderson and Freedberg [3] who showed that the most complete oxidation occurred at those ratios of potassium iodate to AD at which the relative amount of oxidizing agent did not surpass the relative amount of AD. For each portion we used the concentration of oxidizing agent shown in Table 1. The concentration of potassium iodate did not surpass the concentration of AD or NAD by more than 9 times.

COURSE OF THE DETERMINATION

Precipitation of Proteins. To 1 ml of blood taken for the marginal ear vein of a rabbit or from the anterior camera oculi was added an equal amount of a 20% TCA solution for precipitation of the protein, so that in the filtrate would be the free and bound fractions of AD and NAD. In parallel samples of the same volume we ran precipitation of the protein by different amounts of a saturated solution of $(NH_4)_2SO_4$ in order to obtain in the filtrate only a free fraction of AD or NAD. After 20-25 min the precipitate was filtered through a pleated filter, which had first been washed with 10 ml of twice-distilled water. We ran a parallel control on the reagents (C). Further treatment of the filtrates obtained after precipitation by TCA and $(NH_4)_2SO_4$ was carried out in the same way.

In a centrifuge tube with capacity of 10 ml we placed a weight of filtrate (2.5-3.0 ml) and added 1 ml of turbid aluminum hydroxide, 3 drops of saturated $(NH_4)_2SO_4$ solution, and 1 drop of phenolphthalein and made it alkaline with a 4% solution of NaOH to weak color. The samples were stirred continuously with a glass rod, then centrifuged. The centrifugate was poured off and the precipitate was twice washed with 5 ml of slightly alkaline solution. After each washing, the sample was centrifuged. After the last centrifuging, the precipitate was treated with 9 ml of freshly distilled water and 0.2 ml of 0.1 NH_2SO_4; it was well stirred and again centrifuged. The resulting centrifugate was poured into a tube and then divided into 9 tubes of 1 ml each. The control and experimental samples were treated in parallel.

Differentiation. In the first 3 tubes we worked with AD and NAD at concentrations of $1{:}10^{-5}$ to $9{\cdot}10^{-5}$ mmoles/liter; in the second three tubes, from $1{\cdot}10^{-4}$ to $9{\cdot}10^{-4}$ mmoles/liter, and in the last three tubes, from $1{\cdot}10^{-3}$ to $9{\cdot}10^{-3}$ mmoles/liter.

For each series of tubes we used the concentrations of reagents shown in Table 1.

In the first, fourth, and seventh tubes, AD was oxidized. To 1 ml of solution was added one drop of potassium iodate of the corresponding concentration. The solution was carefully mixed on the electric mixer for exactly 4 min. After the mixing the solution was treated with one drop of H_2SO_4 and two drops of KI. The excess iodine was neutralized with three drops of sodium hyposulfite.

In the second, fifth, and eighth tubes we determined the OP. To 1 ml of the solution was added one drop of a 40% neutral solution of formaldehyde, and then one drop of potassium iodate of the corresponding concentration. The addition of the neutral formaldehyde bound the side chain of AD and the specific wave of AD was not shown in the polarograph. This permitted separation of AD from OP, which had properties very similar to those of AD but was not bound by formaldehyde. Then the solution was carefully mixed on the electric mixer for exactly 4 min. The subsequent treatment was the same as for AD.

In the third, sixth, and ninth tubes we set up conditions in which both AD and NAD were oxidized; to 1 ml of solution was added 0.5 ml of a saturated solution of sodium acetate before adding the potassium iodate of the corresponding concentration. Changing the pH of the medium permits polarography of NAD in the presence of AD. The solution was mixed on the electric mixer for 6 min. According to the data of Stock and Hinson [4], this time is required for maximum oxidation of NAD. Subsequent treatment of these samples did not differ from the previous.

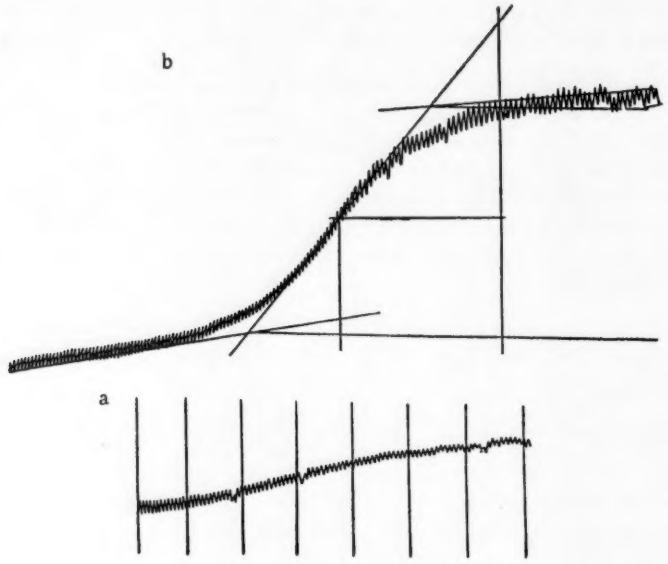
Then to all the solutions we added 5 ml of acetate buffer, pH 4.2-4.3, as a background electrolyte and 2 drops of 0.01% gelatin solution to repress the maximum. Oxygen was displaced by a stream of nitrogen over a period of 6 min. The nitrogen from the gasometer was passed through three successive Tishchenko flasks which contained, respectively, an alkaline pyrogallol solution, 0.1 N H_2SO_4 and twice-distilled water. The addition of solutions in treating the samples was easily carried out by a 5-10 ml dropper held in a special support; this hastened the work. All calculations were referred to 1 ml. The volume of 1 drop was 0.020-0.025 ml.

Polarography of the resulting substances. The study was carried out on the Heyrovsky polarograph system manufactured in 1954. The maximum sensitivity of the galvanometer to the current was $2.2{\cdot}10^{-9}$ a/mm.

To obtain a constant temperature the electrolyzer was placed in a crystallizer with water at 20.0 ± 0.02°. Since a change in temperature of 1° corresponds to a 2% change in wave height, variation of ± 0.02° during the polarographic measurement was quite permissible even if we took the galvanometer readings with an accuracy of 2 ± 0.2 mm.

The capillary used for the measurements had the following characteristics: on the average it contained 1 ml of main solution, 2 drops of 0.01% gelatin, and 5 ml of acetate buffer (pH 4.2-4.3) as the background electrolyte, a total of 6 ml (the volume of the oxidizer was not considered); at a total voltage on the potentiometer of 0.8 V and temperature 20°; τ = 2.73 sec; m = 1.20-1.25 mg/sec; K = 5.2333 $m^{2/3} \tau^{1/6}$.

The mercury used for the polarography was purified in the usual way. The mercury was not distilled because conditions did not require it.



Polarogram of AD solution ($C = 1 \cdot 10^{-4}$ mmole/liter) in acetate buffer.

a) – pH 3.0; b) – pH 4.0

TABLE 4

Test of the Method on Biological Media

Biological medium, ml	Amount of AD or NAD added, in mmole/liter	Amount of AD or NAD determined in mmole/liter	Yield of AD or NAD, %	Biological medium, ml	Amount of AD or NAD added, in mmole/liter	Amount of AD or NAD determined in mmole/liter	Yield of AD or NAD, %
	Blood				Humor of the eye		
	0	0			0	0	0
1.0	0.001	0.00095	95	0.25	0.001	0.00105	105
1.0	0	0	0	0.25	0	0	0
1.0	0.0002	0.0002	100	0.25	0.0001	0.0001	100
1.0	0	0	0	0.25	0	0	0
1.0	0.00004	0.00004	100	0.25	0.00001	0.00001	100

Determination of the diffusion-current constant. For quantitative analysis the dropping electrode was calibrated by taking a polarogram of a known concentration of the substance studied under the same conditions as were used in the analysis. From the results we calculated the diffusion-current constant (K) and the results were expressed graphically.

Analysis of the resulting data showed that at small concentrations of AD there was not a linear relation of the diffusion current (Id) to the concentration of substance (C), which made it difficult to use polarography for the quantitative determination of the substance in solution and contradicted the basic equation of Il'kovich:

$$Id = KC. \quad (1)$$

In order to avoid this we worked with a range of concentrations in the three portions which gave a good linear relation of Id to C.

The values of the diffusion current and its constant for AD and NAD are given in Table 2.

To find the relation C = KH (H is the height of the wave in mm) we prepared solutions in the selected background electrolyte (acetate buffer) in which the concentrations of AD and NAD were changed from $1 \cdot 10^{-5}$ to $9 \cdot 10^{-3}$ mmole/liter.

Table 3 shows that the height of the wave (H) is proportional to the concentration of AD or NAD.

Determination of the diffusion current constant for different concentrations of AD and NAD in acetate buffer showed that the resulting data agree with the basic Il'kovich equation (1) and our solutions can be used for quantitative polarographic analysis of AD and NAD.

It is important to observe that there is a great influence of the pH of the medium on the character of the polarographic wave. At pH 3.0 and below, the polarographic waves of AD and NAD, as the figure shows, cannot be obtained [5].

Although we used a relatively great range of AD and NAD concentrations (from $1 \cdot 10^{-5}$ to $1 \cdot 10^{-3}$ mmole/liter), in all our experiments the waves were well distinguished and could be easily checked. It is known that the chief property of the half-wave potential is its specificity for each given compound and constancy and independence of concentration of oxidizing or reducing ions, if the concentration of the indifferent electrolyte and the temperature of the solution are kept constant. At 20°, anode-cathode polarization, total voltage on the potentiometer 0.8 V, constant concentration of acetate buffer as the indifferent electrolyte, the half-wave potential ($E^1/_2$) for AD was + 0.02 V at pH 3.9-4.1, and for NAD + 0.033 V at pH 4.3-4.5.

RESULTS OF THE DETERMINATION

To test the method we added standard solutions of AD and NAD to blood and aqueous humor before treatment of the samples with TCA.

As Table 4 shows, the biological media do not have any influence on the polarographic determination of AD or NAD. In all cases we found almost 100% yield of added substance.

The sensitivity of the method was $1 \cdot 10^{-5}$ mmole/liter.

The results indicate the very low content of the substance which we study, in blood and humor of the eye.

Direct determination showed the complete absence of AD in both fluids. NAD and OP were found in blood and the humor of the eye in some cases, only in the free form and in concentrations of 1 to 25 mg/liter.

In the analysis of 104 samples of blood and humor of the eye taken at different times from 14 rabbits OP was detected respectively in 55 and 52 samples, and NAD in 57 and 49. Correlation between content and presence of OP and NAD in these fluids was not established.

SUMMARY

We have worked out a differential polarographic method for the determination of AD, NAD, and OP. The sensitivity of the method is $1 \cdot 10^{-5}$ mmole/liter.

When we use this method to study the blood and humor of the eye in rabbits, AD is not found at all. NAD and OP are found in some cases in blood and humor of the eye, only in the free form and in concentrations from 1 to 25 mg/liter.

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TRANSFORMATIONS OF SEDOHEPTULOSE-C^{14} IN THE LEAVES OF BEANS, TOBACCO, AND SEDUM SPECTABILE

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Sedoheptulose was first obtained in 1917 in the plant Sedum spectabile [1]. However, it was only a few years ago that its phosphate ester was found among the early intermediate products formed in the fixation of $C^{14}O_2$ during photosynthesis [2, 3]. It was then shown that sedoheptulose-7-phosphate was one of the main intermediate products of photosynthesis, since because of its reactions it became possible to regenerate ribulose diphosphate which is a CO_2 acceptor in photosynthesis [4-6].

On the basis of work carried out in recent years it can be assumed that the regeneration of ribulose diphosphate occurs as follows: sedoheptulose-7-phosphate in the presence of transketolase [7, 8] splits out a fragment of "active" glycolaldehyde and is transformed into glyceraldehyde-3-phosphate with the formation of ribulose-5-phosphate. The ribose-5-phosphate which remains after "active" glycolaldehyde splits out is isomerized in the presence of phosphopentoisomerase to give a second molecule of ribulose-5-phosphate [9, 10]. In the presence of ATP and the enzyme phosphopentokinase obtained from spinach leaves [10], ribulose-5-phosphate is changed to the CO_2 acceptor, ribulosediphosphate. It is natural that the sedoheptulose-7-phosphate because of its important role in photosynthesis should attract much attention, and the question of its changes in this process is of interest.

This question was studied in detail in the work of Tolbert and Zill [11]. They sucked a solution of labeled sedoheptulose through the petioles of cut leaves of sugar beet, tobacco, and barley and showed that the resulting labeled products did not differ in principle in the plants which they studied. Among the products of photosynthesis formed after 11 min and longer times, an important place was occupied by sucrose, glyceric acid, alanine, and erythrose phosphate. On the basis of this they assumed the presence of a rupture between the third and fourth carbon atoms of sedoheptulose. In the dark, sedoheptulose was transformed chiefly into glutamic, aspartic, and succinic acids.

We decided to make a very close study of the transformations of sedoheptulose in the shortest possible time under different conditions. Since it had been shown that the transformations of the early intermediate products of photosynthesis also depend to a considerable degree on the characteristics of the total metabolism of the substances [12], we used different types of plants for the experiments: beans, tobacco, and Sedum spectabile. The latter species of plant, as is known, is distinguished by its ability to accumulate a considerable amount of sedoheptulose, and therefore we could expect that this plant would show a different set of reactions.

The labeled sedoheptulose was obtained from the leaves of Sedum spectabile. The young plants were placed in a closed glass chamber in which was placed $C^{14}O_2$. The air in the chamber was constantly changed by a special ventilator. The plants were kept in the presence of radioactive carbon dioxide in the air for about a day with illumination with a 500 W lamp at a distance of 80 cm.

The radioactive sedoheptulose was isolated as a sirup by the method of Pratt et al. [13]. In order to free the sirup from contaminating labeled sugars, we fermented it with a pure culture of brewers yeast, Sacharomyces-Carlsbergensis. The yeast suspension after fermentation was treated with lead acetate. The resulting white precipitate was filtered off and hydrogen sulfide was passed in until all the lead had precipitated. The precipitate of lead sulfide was filtered off and the solution was treated with cationite KU-1 and anionite AN-2F.

After treatment with the ion exchange resins, the preparation was tested for purity by paper chromatography and radioautography.

Neither labeled sucrose nor glucose were found on the chromatograms or the radioautographs. On the radioautographs, besides sedoheptulose we found two weak radioactive spots, well separated from each other, which corresponded to two unknown substances. These substances were located far from sedoheptulose. It could be freed from them easily by paper chromatography. For this purpose the preparation was placed on filter paper and after chromatography the position of the radioactive sedoheptulose was determined on an X-ray film. The sedoheptulose zone, marked on the chromatogram, was cut out, eluted with water, and the solution was evaporated in a vacuum at room temperature. The resulting pure sedoheptulose-C^{14} had a specific radioactivity of 0.022 μC/ mg and had an approximately even distribution of the labeled atoms.

TABLE 1

Percent Distribution of Radioactivity in the Products of the Transformation of Sedoheptulose-C^{14}, Distributed in Different Fractions.

Plant	Exposure in min and experimental conditions	In 80% alcohol fraction		In fraction insoluble in 80% alchohol		In petroleum ether fraction	
		Light	Dark	Light	Dark	Light	Dark
Bean	2, air	99.20	99.18	none	none	0.80 0.50	0.82 −*
"	3 "	99.50	−*	none	none	0.54	0.33
"	5 "	99.34	99.67	0.12	none	0.22	−*
"	5, nitrogen	98.58	−*	0.20	−*	1.39	−*
"	10, air	98.40	−*	0.21	−*	not determ.	1.19
"	30 "	98.38	98.64	0.65	0.17	not determ.	
"	120 air	97.10	98.33	1.92	0.51	not determ.	1.16
Tobacco	2, "	99.75	~100.00	none	none	not determ.	
"	10, "	99.72	~100.00	0.10	none	0.25	none
Sedum spectabile	2, "	99.93	−*	0.05	−*	0.18	none
"	10, "	99.94	−*	0.06	−*	0.02 none	−* −*

* At the given exposure the experiments were carried out only in the light.

In the experiments in the light the plants were first illuminated with a 500 W lamp for 30 min, and in the experiments in the dark, the plants were kept for the same time in the dark. Disks with a diameter of 14 mm were cut from the leaves of the experimental plants. It has been shown [14] that when such disks are infiltrated, they are capable of carrying out the normal photosynthetic process. An 0.8% solution of labeled sedoheptulose was infiltrated into the leaf disks. After infiltration, the leaf disks of the experiments in the light were illuminated for different periods under the conditions of the previous illumination of the plants, and the disks of the experiments in the dark were kept for a corresponding period in the dark. Then the disks were fixed with hot 80% alcohol, into which most of the radioactivity passed. Extraction with alcohol was repeated until all the soluble substances were removed. The alcohol extract was evaporated in a vacuum at room temperature to a thick sirup, from which pigments and lipids were removed with petroleum ether. The radioactivity of each fraction was measured in the usual way with a counter. The results are given in Table 1.

The results of Table 1 show that under all the experimental conditions, almost all the radioactivity was contained in the alcohol fraction. With increasing exposure in all the plants there was a regular increase in radioactivity in the high molecular-weight compounds, insoluble in 80% alcohol. The formation of these compounds in the light occurred faster than in the dark. The considerable radioactivity in the fraction of substances soluble in petroleum ether was interesting. The radioactivity in this fraction in our experiments with short exposure was several times greater than the corresponding photosynthetic fixation of $C^{14}O_2$.

We can assume that the considerable inclusion of radioactivity in the fraction of substances soluble in petroleum ether after infiltration of radioactive sedoheptulose into the leaf is connected with the easy splitting from the sedoheptulose of the two carbon fragments, which, as is known, can easily be converted by the plant into fats, pigments, and other substances which occur in this fraction [15-17].

In order to explain through what substances the sedoheptulose chiefly passes in its transformations in the leaf, we submitted the alcohol fraction to a detailed chromatographic analysis.

For the two-phase chromatogram we used as solvents, phenol saturated with 0.1 N formic acid, and butanol-acetic acid-water (78:20:50). For the one-phase chromatogram we used the solvent butanol-acetic acid-water (78:20:50:). In the separation of some amino acids we used phenol-phosphate buffer (1:1) with pH 12. After exposing the chromatogram on X-ray film for three-six months, we obtained a radioautograph and determined the radioactivity of the spots which formed.

TABLE 2

Distribution of Radioactivity in the Alcohol Fraction from Bean Leaves after Chromatography, in %

Exposure in min	Light		Dark	
	Sedo-heptulose	Sucrose	Sedo-heptulose	Sucrose
2	88.5	0	~100	0
5	87.0	Trace	~100	0
30	79.2	10.1	~100	0
120	69.1	17.2	97	2.2

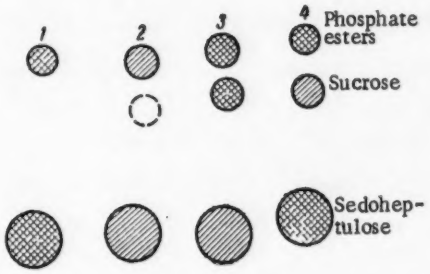

Figure. Radioautograph with chromatogram of alcohol fraction from beans. Experiment of Feb. 8, 1957. In the light: 1 – 2 min; 2 – 5 min; 3 – 30 min. 4 – 2 hours.

It was shown that the transformation of sedoheptulose goes chiefly toward formation of sucrose, whose spots have considerable radioactivity (Table 2).

Radioactivity was also found in the spots from the phosphate esters (Fig.). Weak radioactivity was observed in malic acid, serine, glycine and an unknown substance whose spot occurred on the chromatogram in the region of sucrose.

Table 2 shows that the light formation of sucrose from sedoheptulose in bean leaves begins after 5 min exposure. After 30 min its amount is considerably increased, and then it begins to increase more slowly, reaching 17% in 2 hours, that is, its greatest value from transformation of sedoheptulose. In the dark, formation of sucrose goes much more slowly, and can be clearly observed only after a 2-hour exposure. This fact evidently indicates that when sucrose is formed from sedoheptulose the reactions of the carbohydrate photosynthetic cycle are used, those which require the presence of light. The formation of sucrose as the chief product of the transformation of sedoheptulose in the photosynthesizing leaf agrees with the assumption that it is the first free sugar which accumulates in photosynthesis [18]. As shown above, along with the sucrose spot on the radioautograph there is a well-developed spot in the zone of the phosphate esters, monophosphates. Judging by the position of this spot we can assume that it corresponds to sedoheptulose monophosphate. The formation of this ester precedes the formation of sucrose and is evidently the initial stage in the transformation of sedoheptulose. The figure shows that small amounts of the phosphate ester are already present after 2 min exposure. It appears more intense after 5 min exposure, and at 30 min and 2 hour exposure its quantity gradually falls. Except for the substances mentioned, in the species of plants which we studied we did not find any other intermediate products of sedoheptulose transformation.

The use in similar experiments of sedoheptulose with considerably higher specific activity would permit a more detailed explanation of the path of its transformations.

In view of the presence of a large amount of sedoheptulose in the leaves of Sedum spectabile, the isotope was diluted. Evidently, therefore, the transformation of sedoheptulose in the leaves of this plant takes place less intensively (Table 1). No other peculiarities in the transformation of sedoheptulose in the leaves of any of the species of plants which we studied were observed.

SUMMARY

The transformation of evenly labeled sedoheptulose infiltrated into leaves occurs chiefly with formation of sucrose. Since this transformation occurs much faster in the light, we can assume that it occurs through the reactions of the carbohydrate photosynthetic cycle. Even 2 min after infiltration some phosphate esters are formed which are located in the chromatographic zone of sedoheptulose monophosphate. The formation of this ester is evidently the initial step in the transformation of sedoheptulose. As a result of the transformation of labeled sedoheptulose, we have observed a weak activity in malic acid, serine, and glycine. We have observed some radioactivity in the fraction of high molecular-weight compounds, insoluble in 80% alcohol, and also in the fraction of substances soluble in petroleum ether.

No difference has been seen in the transformations of sedoheptulose in the leaves of beans, tobacco, or Sedum spectabile.

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NEW DATA ON THE FRACTIONATION OF CELL NUCLEI OF RAT LIVERS AND THE CHEMICAL COMPOSITION OF NUCLEAR STRUCTURES

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In previous work from our laboratory we have shown that in cell nuclei isolated from different tissues in a neutral water medium which contains citrate, we can successively remove a nucleoprotein fraction soluble in 1 M NaCl, a fraction of acid protein extracted by dilute alkali solution, and can separate a fraction of residual protein which is not removed by ordinary solvents. These protein fractions differ from each other in amino acid composition, content of nucleic acids, and physicochemical properties [1-5].

The residual protein of the liver cell nuclei was identified by Soudek and Benes [6] as the material of the nuclear membrane, while the other two fractions and the globulin fraction described later by other authors could be assigned to some locality or function in the nuclei only on the basis of indirect cytochemical data [7, 8].

In the work of one of us [9] we carried out successive extraction of thin, unfixed sections of different tissues. The 0.14 M NaCl which dissolved the globulin fraction extracted the protein and RNA not bound to chromatin or nucleolus but which appeared in the nuclei in the non-extracted sections, 1 M NaCl removed all the desoxyribonucleoprotein (DNP) of the nucleus. The ribonucleoproteins of the nucleolus and the structures which resemble the residual chromosones of Mirsky and Ris [10] are retained in this treatment, but are removed after treatment with dilute alkali. On this basis it was concluded that the globulin fraction includes the nuclear sap, the nucleoprotein fraction ,the main mass of chromatin, and the acid protein,the nucleoli and remaining chromatin.

However, that part of the nuclear mass which occurs on the nucleolus and residual chromatin did not correspond quantitatively to the high percent content of acid protein in the cell nuclei (40-50%) [1, 7, 11, 12]. The reason for this lack of agreement could be that the isolation of the nuclei in dilute citric acid at pH value close to neutral, using a Waring blendor, as the later work of Dounce, O'Connel, and Monty [13, 14] showed, could destroy themitochondria and liberate from them proteolytic and nucleolytic enzymes which could split non-histone proteins from DNA.

These authors consider that the outward evidence of this autolytic change is the loss of ability to form a gel when the nuclear suspension is made alkaline or is placed in a 5-10% salt solution. According to Dounce, the extractability of DNP by salt solutions is also a result of autolytic change [15]. However, in the above-mentioned cytochemical work [9] 1 M NaCl fully removed DNP from the nuclei through which a section was cut although the method of treatment excluded the possibility of enzymatic autolysis.

In the present work we describe the results of fractionation of the nuclei of rat livers after isolation under conditions for maximum retention of their native state, taking account of the above new data and some discrepancies between the results obtained in our laboratory and those in the laboratory of Dounce.

METHODS

For isolation of the nuclei we considered it necessary to fulfill the following requirements: 1) prevention of destruction of the mitochondria, which would liberate proteases and nucleases; for this purpose we replaced the previously used Waring blendor by a glass bacterial mill which operated at a small speed and we added potassium glycerophosphate to the suspension medium; this has a strong stabilizing action on mitochondria [16]; 2) a more

complete purification from cytoplasmic contaminants which could change the quantitative ratio between the protein fractions of the nucleus. For this we used a concentrated sucrose solution according to Chauveau [17] which in one centrifuging permits separation of the nuclei from the cytoplasmic particles and whole cells which come to the surface since they have a density less than the density of the medium, and 3) removal of the possibility of a secondary binding of proteins by DNA, for which we carried out the separation in a neutral medium.

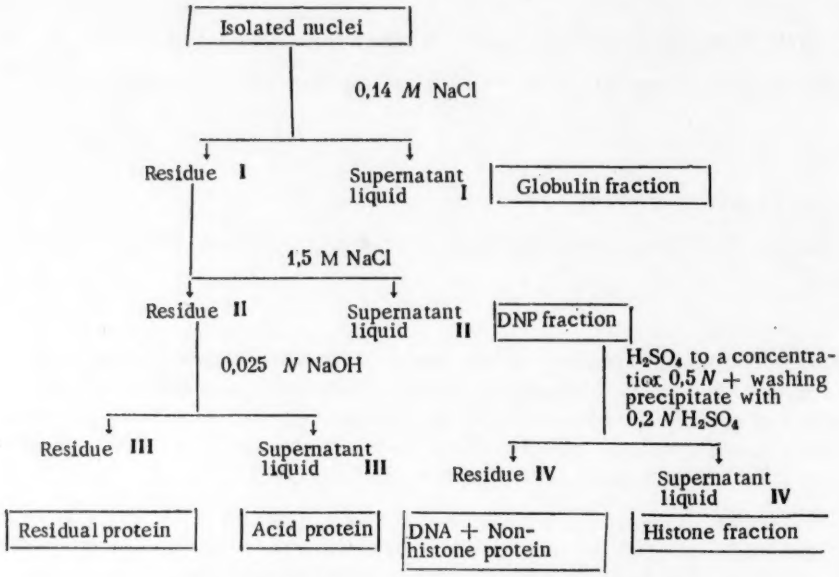

Fig. 1. Scheme of the fractionation of nuclear proteins

Isolation of the nuclei. The isolation of the nuclei and their subsequent fractionation were carried out in a cold room at 2-4°. The rat was decapitated. The liver was perfused through the inferior vein cava at first with 0.14 M NaCl, then with 0.25 M sucrose. 8 g of perfused liver, after grinding in a glass homogenizer with vertical pestle was suspended in 120 ml of solution containing 0.065 M potassium glycerophosphate, pH 6.9-7.0, and sucrose, added in amount needed to give a density of the solution at 4° of 1.273 (about 2.2 M). The suspension was passed twice through a bacterial mill whose inner cone rotated at 1000 to 1500 r.p.m. The latter was cooled with a mixture of ice and salt. The homogenate was contrifuged for 1.5 hours at 18000 g with cooling. Then the nuclei, not containing cytoplasmic impurities or undestroyed cells, settled to the bottom of the centrifuge tube. When the nuclei were isolated in a medium with $d < 1.270$, the precipitate was contaminated with cytoplasmic impurities; at $d > 1.275$ most of the nuclei floated. From 8 g of crude liver we were able to get about 30 mg of dry nuclear substances.

To test the ability of the nuclei to form gels we added several drops of 0.025 M NaOH or different volumes of 2 M NaCl to their suspensions in water.

Fractionation of the nuclei. The course of the fractionation of the cell nuclei is given in the scheme (Fig. 1). For removal of the globulin fracion the isolated nuclei were twice extracted with 0.14 M NaCl for 40 min in a Potter-Elvehjem glass homogenizer. The residue was homogenized with 1.5 M NaCl and mixed overnight with a magnetic stirrer. The resulting viscous solution was separated by centrifuging at 18000 g for 2 hours. The precipitate was again extracted with 1.5 M NaCl in the same way. The insoluble residue was again treated with a new portion of 1.5 M NaCl in the Waring blendor for 1 min and again the insoluble residue was separated. The latter was extracted three times with 0.025 N NaOH to obtain the acid protein fraction. The residual protein remained in the precipitate. The combined extracts in 1.5 M NaCl (DNP solution) were precipitated with 0.33 of the volume of 2 N H_2SO_4. The precipitate was separated by centrifuging at 18000 g and was washed with 0.2 N H2SO4. This last extract contained the histone fraction. The washed precipitate contained the non-histone protein in a complex with DNA. In order to obtain a sufficient quantity of material, we separated the nuclei repeatedly from two to three liver portions. Bach portion was at once extracted with 0.14 M NaCl and transferred to 1.5 M NaCl. After this all the portions were combined.

Analytical methods and cytological control. In each fraction we determined the nitrogen by micro-Kjeldahl. Then the proteins and the nucleic acids were precipitated with cold TCA at a final concentration of 7%. After washing with 5% TCA, twice with alcohol, and with ether, the precipitate was hydrolyzed for 18 hours at 37° in 0.5 N KOH and the RNA and DNA fractions were separated according to Schmidt and Tannhauser [18], after which the RNA was determined according to Meibaum [19] by its phosphorus, the DNA according to Dishe [20] and Webb [21] by its phosphorus. Phosphorus was determined according to King [22].

Cytological study of the results of the extractions was carried out by a methyl green-pyronin stain [23] and in a number of cases with bromophenol blue [24] and according to Feulgen.

RESULTS AND DISCUSSION

Description of the Isolated Nuclei. The nuclei isolated by the above method formed a gel in an alkaline medium. Extraction of DNA with salt solution occurred with great difficulty. Thus, both signs were shown which, according to Dounce, characterize nuclei which have not undergone autolytic change, and in which the bond between DNA and non-histone proteins is not broken.

In the phase-contrast microscope the nuclei look "whole"; the nuclear contents appear homogeneous except for the clearly traced dark nucleoli. The same picture appears when the nuclei suspension in sucrose is stained with dilute methylene blue (Fig. 2, a). In the microscopic study we did not see any cytoplasmic contaminants or unbroken cells.

TABLE 1

Percent Content of Protein Fractions in Rat Liver Nuclei

Fraction	Experiment No.										Average and mean error
	1		2		3		4		5		
Globulin fraction	19,5		17,6		20,6		23,5		21,0		20,4±0,98
RNA in the experiment		0,9		1,4		0,8		0,6		1,1	
DNP fraction	70,6		72,0		69,7		68,2		66,8		69,5±0,91
In the same expt. DNA		17,4		17,9		19,2		16,6		18,8	18,0±0,46
Non-histone protein		19,2		18,0		15,6		18,0		13,7	16,9±0,98
Histone		33,6		35,7		34,4		33,2		33,8	34,1±0,45
Acid protein	5,7		5,8		5,1		5,6		5,6		5,6±0,12
RNA In the same experiment		1,0		0,9		0,8		0,7		1,0	
Residual protein	4,2		4,6		4,6		2,7		6,6		4,5±0,62

After isolation of the nuclei by the unmodified method of Chauveau, Moulé, and Rouiller [17] the ability to form a gel becomes weaker. The cytological picture seen in the extraction process [9] is also much less clear.

In nuclei isolated by the method described in the present work the content of DNA is 8.0 ± 0.46% and RNA 2.28 ± 0.19% of the total proteins and nucleic acids of the nuclei, calculated by nitrogen content [11]. Thus the ratio RNA/DNA is 0.127. This figure agrees approximately with the data of Chauveau [17] for rat liver nuclei (DNA 17.8, RNA 2.88%) and is only a little above the data of Mirsky [8] for liver nuclei of calves and horse isolated in an anhydrous medium (which excludes loss of water soluble proteins). The lower values for DNA content and higher ones for RNA obtained by Dounce [7] probably depend to a considerable extent on cytoplasmic contaminants.

In view of these reports, we have assumed that the nuclei which we isolated retain fully or almost fully the proteins and nucleic acids which composed them. The portion of the starting material lost, if there was any, could only have been in the globulin fraction. The high degree of purity of the nuclei makes it improbable that there were cytoplasmic contaminants in any fraction. The neutral pH in the isolation of the nuclei did not supply a favorable set of conditions for secondary binding of cytoplasmic proteins by the nucleic acids of the nuclei.

When a fixed smear was stained with methyl green-pyronin, the structures of the nuclei did not differ from those observed in frozen sections (Fig. 2, b).

TABLE 2

Content of Nuclear Proteins Calculated per Unit of DNA

Fraction	Experiment No.					Average and mean error
	1	2	3	4	5	
Globulin	1,11	0,98	1,07	1,40	1,10	1,13±0,070
Non-histone protein	1,09	1,01	0,81	1,07	0,72	0,94±0,074
Histone	1,91	1,99	1,78	1,97	1,77	1,88±0,046
Acid protein	0,33	0,35	0,26	0,33	0,29	0,31±0,038
Residual protein	0,25	0,28	0,24	0,16	0,35	0,26±0,076

Results of the Extraction. After extraction with 0.14 M NaCl, the nuclei which we isolated like those in sections obtained with the freezing microtome [9], retained proteins and nucleic acids which corresponded cytologically to chromatin, and the nuclear sap was removed. The addition of 1.5 M NaCl caused formation of a nuclear gel. As a result of the first centrifuging, after the extraction overnight, the precipitate retained its gelatinous form and held a considerable percent of the original DNA. Microscopically the nuclear gel appeared as a homogeneous mass of DNP, stained by methyl green, with numerous red (pyronine) nucleoli and residual chromosomes dispersed in it (Fig. 3). Repeated extractions removed almost all the DNP so that the smears contained almost no material stained by methyl green or according to Feulgen. There then remained in the preparation many nucleoli of different diameters which often had a regular round form, and residual chromosomes in the form of fibers and fine green structures (Fig. 4). They were clearly stained by pyronine and less clearly by bromophenol blue. A small quantity of homogeneous protein formations colored pale red by bromophenolblue also appeared. The extraction by dilute alkali fully removed RNA and the proteins of the nucleoli and residual chromosomes. This confirmed that the acid protein fraction contained only RNA and the proteins of the nucleoli and residual chromosomes.

To summarize the cytological findings on the course of the extraction from nuclei isolated under conditions which made it possible to retain the native state, we can conclude that the chemical fractionation agrees with the cytological fractionation and each fraction corresponds to one or another internal structure.

In passing it is of some interest to mention some characteristic features of the nucleoprotein fraction. When the viscous DNP solution in 1.5 M NaCl was diluted 6-8 times with water, the addition of acid, alcohol, or acetone precipitated a characteristic fibrous mass, distinctively a complex which contained high molecular-weight DNA. However, after the solution had been treated mechanically for 1 min in a Waring blendor, this behavior disappeared and all the precipitants mentioned, including dilution by water, caused the appearance of a fine-grained, amorphous precipitate. Thus, depending on the mechanical treatment of the solution, DNP can be obtained in a fibrous or an amorphous state. This observation indicates the presence in the DNP solution of an internal structure (or organization) which is sensitive to mechanical action. In such mechanically treated solutions of DNP after 8 days standing at 0° the ability to form fibers when precipitated is not restored. DNP solutions which have not been treated mechanically do not lose the ability to form fibers on standing.

Quantitative Relation between Protein Fractions of Cell Nuclei and Their Composition. The amount of any fraction in the cell nuclei is expressed as the percent of the sum total of the proteins and nucleic acids of the nuclei (Table 1) calculated from the total nitrogen content [11]. For this the content of nitrogen in the histones is calculated as 17% [8] and in the other proteins and nucleic acids as 16%. Also, in consideration of the constant composition of DNA in the cell nuclei of a given species, we have made calculations DNA units (Table 2).

The globulin protein, including the proteins and RNA of "nuclear sap", makes up about 20%. Calculated per unit of DNA it is evident that its amount varies considerably (from 0.98 to 1.40). The possibility is not excluded that part of the globulin fraction is lost in the isolation of the nuclei. RNA is found in the globulin fraction with a content which varies from 2.6 to 8%. This variation probably depends on the heterogeneity of the globulin fraction which, according to the literature, [7, 25, 26], contains both simple soluble proteins and ribonucleoprotein granules.

TABLE 3

Composition of the "Chromosome Proteins"

Fraction	Components included in the fraction	Percent content	
DNP	DNA	24.2	90.9
	Non-histone protein	21.2	
	Histone	45.5	
Acid protein	RNA	2.2	9.1
	Protein	6.9	

The nucleoprotein, or more accurately, the desoxyribonucleoprotein (DNP), fraction makes up about 70% of the nuclear mass and only 5.6% of this comes from acid proteins. These results differ from those obtained by an almost identical fractionation procedure, but using nuclei separated under other conditions [1, 3, 11, 12], and from the results of a study in which the scheme of fractionation did not permit separation of the different proteins of a non-histone character [7]. The chief difference is that in these cases the values given for the content of acid protein or the fraction which corresponds to it were 40-50% of the nuclear substance.

Further analysis showed that about $^1/_3$ of the protein component in our DNP fraction consisted of non-histone protein. It is suggested that this result depends on preserving the easily ruptured bond between DNA and the non-histone protein of DNP, so that the latter does not appear in the acid protein fraction, but in the DNP fraction. It may also be that in the acid protein fraction there is some other type of non-histone protein bound to the nucleoli and residual chromosomes.

Thus the DNP fraction consists of DNA, histone, and non-histone proteins in the approximate ratio: 1: 2: 1. The histone content in the liver nuclei is 34.1 ± 0.30% and the variation from experiment to experiment lies within very narrow limits as is also the case for the histone/DNA ratio. The variation in the content of the non-histone protein is greater.

This procedure for the isolation of the histone fraction gives good, reproducible results. It can be considered to be based on the following considerations: according to Mirsky and Ris [27] histones can be fully removed from isolated chromosomes by acidified 1 M NaCl. On the other hand, histone free from acid protein can be fully extracted with 0.5 N H_2SO_4 but no with HCl [28, 29].

Aside from the components mentioned above, we observed in the DNP fraction a small amount of RNA. Since the solution of DNP was very viscous and the precipitation of insoluble particles from it was dificult, we assumed that RNA came from residual chromosomes which did not fully settle out under our conditions of centrifuging (18000 g). To test this assumption we further centrifuged the solution of DNP in 1.5 M NaCl at 18000 g for 4 hours. About $^2/_3$ of the RNA from the DNP fraction on this, passed into the precipitate and the ratio of RNA/protein in the precipitate was the same as in the acid protein. We are therefore inclined to believe that the presence of RNA in the DNP fraction depends on the contamination of this fraction by residual chromosomes.

The acid protein, containing the RNA and the protein of the nucleoli and residual chromosomes, makes up about 5.6% of the nuclei and contains, on the average, 15.6% RNA (in separate experiments, 17.5, 15.2, 15.5, 11.3, 18.9%). The high percent of RNA corresponds to data obtained with ultraviolet spectrophotometry of nucleoli of nerve tissue [30] and indicates that nucleolus material as a whole enters the fraction of the acid protein. Besides RNA, the acid protein contains some DNA. With an increased number of extractions its percent falls sharply. In cytological analysis of the preparations stained by the Feulgen method, we see that DNA is not bound with the nu-

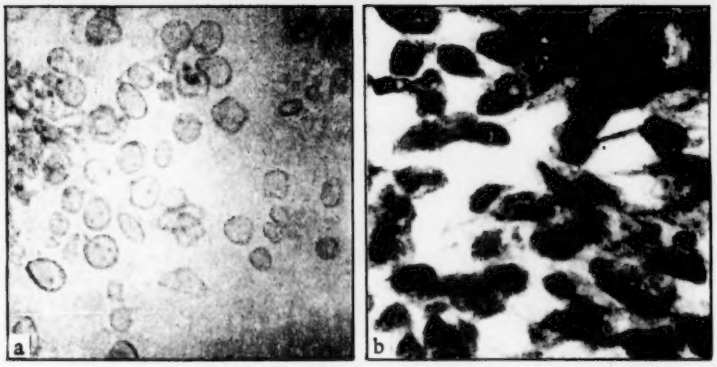

Fig. 2. Isolated nuclei. a) Stained with methylene blue; b) stained with pyronine-methyl green.

Fig. 3. Nuclear gel after first extraction with 1.5 M NaCl:
1) desoxyribonucleoprotein fibers precipitated by alcoholic fixation; 2) nucleoli.

Fig. 4. Nucleoli and residual chromosomes after removal of DNP fraction:
1) residual chromosomes; 2) nucleoli.

cleoli or residual chromosomes, but forms a background in separate parts of the preparation. Thus, the presence of DNA most quickly of all indicates insufficiently complete extraction of the DNP fraction; evidently it is absent in the nucleoli and residual chromosomes. Repeated extractions led to an increased volume of the extract and so, as a rule, were not carried out. The high values for the DNA content in isolated liver nucleoli, along with the very low RNA percent obtained in the laboratory of Dounce [7, 31] are considered by us to be due to the fact that in these experiments they isolated, preferably, material from the perinucleolar chromatin, while the RNA of the nucleoli themselves was lost to a considerable extent in the wash water.

The fraction of residual protein makes up about 4.5% of the nuclear material and lacks nucleic acids.

The relation of the chemical components in the chromosomes and in the nucleoli is of great interest. They are evidently made up of the DNP fraction and the acid protein [7-9]. The DNP fraction, including the complex of DNA with histone, and non-histone proteins, forms the chief mass of the chromosomes (chromatin, including the perinucleolar chromatin), and the acid protein, which contains a high percentage of RNA, makes up the main material of the nucleoli and residual chromosomes. The latter are, as it were, the skeletons of the chromosomes. Both cytology and solubility indicate considerable similarity between the nucleoli and the residual chromosomes. Since there are data [7] which show the occurrence of nucleoli in definite parts of the chromosomes, we can assume that they are derivatives of the residual chromosomes. The structure of the nucleolar-perinucleolar chromatin in this case will correspond to the structure of the residual chromosomes. Corresponding to this (Table 3) we give the percentage of components produced only from the initial DNP and acid protein with account taken of their cross contamination, but after deduction of the fraction of residual protein and the globulin fraction.

The resulting values can definitely characterize the chemical components of the chromosome-nucleolus apparatus of the cell nuclei of rat livers.

SUMMARY

The described method for obtaining cell nuclei from rat livers is a modification of the method of Chauveau, Moule, and Rouiller. The nuclei isolated by this method do not contain cytoplasmic admixtures and retain the properties of the native state. Treatment of the nuclei so isolated with solutions of sodium chloride and dilute alkali by the somewhat modified method of Zbarskii and Debov permits their separation into fractions which correspond to definite cytological structures of the nucleus.

The globulin fraction, removed by 0.14 M NaCl, comprises about 20% of the total nuclear protein and nucleic acid and contains 2.6-8.0% RNA. It corresponds to the nuclear sap.

Desoxyribonucleoprotein fraction, removed by 1.5 M NaCl, makes up about 70% of the nuclear substance. Of it, about 18% appears as DNA, 34% as histone, and 17% as non-histone protein. This fraction corresponds to the main mass of chromosomes (chromatin). Depending on the method of treatment of the solution we can obtain DNP as a fiber or in a fine-grained, amorphous state.

The acid protein, removed by dilute alkali, makes up about 5.6%. It is rich in RNA (15-17%). This fraction contains RNA and protein of the nucleolus and residual chromosomes.

It is assumed that the nucleoli are derivatives of the residual chromosomes.

The residual protein consists of 4-5% of the nuclear mass and lacks nucleic acids.

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A STUDY OF FREE AND BOUND LIPID FRACTIONS OF THE BRUCELLA TYPE SUIS 44 BY THE METHOD OF CHROMATOGRAPHY

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In a previous communication [1] we established the content of phosphorus, nitrogen, and carbohydrate in all lipid fractions of brucellae. These results fully agreed with the fact that lipids in the bacterial cell do not occur in the free state, but are closely bound to other protoplasmic substances.

At the present time there is already a considerable amount of work which confirms the existence of complex compounds of lipids with carbohydates and proteins.

Also, a number of studies [2, 3] have shown the presence of carbohydrates as structural components of lipids.

The present work is devoted to a more detailed investigation of the carbohydrate and nitrogen compounds of lipids.

EXPERIMENTAL

As the material for the investigation we used three-day cultures of brucella type suis 44, grown on Martin broth. The bacterial mass was separated from the nutritive medium by centrifuging, washing with physiological saline and distilled water and drying in a thermostat at 37°.

Obtaining the Different Lipid Fractions. The dry bacterial mass was successively extracted with ether, benzene, and chloroform in a Soxhlet apparatus for 10 hours with each of these solvents. The ether and benzene extracts were combined and after removal of the solvent in a current of carbon dioxide were dried in a vacuum desiccator; these were free lipids, soluble in ether and benzene.

After the same treatment the chloroform extract gave the free lipids which were chloroform soluble.

The ether-benzene extract was further separated by acetone into fractions soluble and insoluble in acetone. Thus the free lipids gave chloroform soluble, acetone soluble, and acetone insoluble fractions.

To obtain the bound lipids of the bacterial mass which remained after separation of the free lipids we treated it with a mixture of organic solvents and concentrated hydrochloric acid (300 ml of ethyl alcohol, 450 ml of ether, and 13.5 ml of hydrochloric acid).

In order to determine the chemical nature of the different components which occur in the free and bound lipids, we submitted the resulting preparations to alkaline hydrolysis. A sample of about 200 mg of each fraction was saponified with an 0.5 N alcoholic solution of sodium hydroxide for 30 min on a boiling water bath. The cooled hydrolyzate was acidified with 20% acetic acid to an acid reaction to methyl orange. The free fatty acids were extracted by ether. The ether extract served as the material for the study of the fatty acids. The acidified water solution was evaporated dry on the water bath. The dry residue was washed several times with ether and then dissolved in a small amount of distilled water. The water solution of the unsaponifiable fraction was passed through an ion-exchange column with a strongly acid resin KU-1 to remove cations. The column was repeatedly washed with distilled water. The filtrate and wash water were again evaporated dry. The dry residue was used for qualitative study of the carbohydrates by the chromatographic method.

Each dry residue obtained from one of the lipid fractions was dissolved in 0.2 ml of distilled water and 0.05-0.03 ml portions were placed on the chromatogram.

Chromatographic separation was carried out in a mixture of butanol-acetic acid-water (40:10:50:) by the ascending method.

The substances from each fraction obtained after alkaline hydrolysis were treated by the above process and divided into two parts, in one of which we made a direct study of the carbohydrates, and the other of which was submitted to a further acid hydrolysis with N HCl for 4 hours.

TABLE 1

Carbohydrate Composition in Different Lipid Fractions before and after acid Hydrolysis

Name of lipid fraction	Carbohydrate composition	
	Before hydrolysis	After hydrolysis
Acetone soluble	Hexose, arabinose, and disaccharide	Hexose, arabinose (clearly), unidentified spot
Acetone insoluble	Glycerol (clearly), arabinose, hexose, disaccharide	Hexose, arabinose
Chloroform soluble	Glycerol, hexose (weakly), disaccharide	Hexose, arabinose
Bound lipids	Glycerol, arabinose (weakly), disaccharide	Glycerol (clearly), hexose, arabinose

On the chromatograms of these fractions before acid hydrolysis, we noted small amounts of hexose (glucose or galactose), arabinose, disaccharide, and considerable amounts of glycerol (Table 1).

The identification of the hexoses was difficult because glucose and galactose have very close R_f values (0.18 and 0.16 respectively).

In separate samples of the acetone soluble fraction we ran a qualitative reaction for hexoseamine. For this, 96 mg of substance was hydrolyzed by N HCl on the boiling water bath for 6 hours. We ran the reaction for hexoseamine with p-dimethylaminobenzaldehyde on the hydrolyzate. As a control we used casein treated in the same way.

A positive reaction with p-dimethylaminobenzaldehyde permitted us to assume the presence of hexoseamine in the fraction studied.

In other fractions this study was not carried out because of an insufficient amount of the material studied.

After acid hydrolysis the spot which corresponded to disaccharides on the chromatogram was retained only in the acetone-soluble fraction and perhaps belonged to the hexoseamine. The spots corresponding to hexose and arabinose were strengthened. This gave us reason to suppose that these sugars were present in the lipids as polysaccharides (Fig. 1).

For a solution of the problem of whether the observed carbohydrate and glycerol mixture was bound to the lipids, we studied the unsaponified and unhydrolyzed acid fraction of the lipids.

We did not find spots on the chromatogram which corresponded to carbohydrates and glycerol. This confirmed our assumption that the carbohydrates were bound to the lipids. However, the material which we had at our disposal did not permit us to say definitely whether they were a structural part of the lipids or formed an adorption complex with them.

Study of the Fatty Acids. The ether extract obtained after alkaline hydrolysis was used for the investigation of the fatty acids. The very limited amount of material which we possessed allowed us to use only the chromatographic method of investigation. There were difficulties in the chromatographic study of the higher fatty acids, and so we worked out a suitable method [5].

Fig. 1

Fig. 2

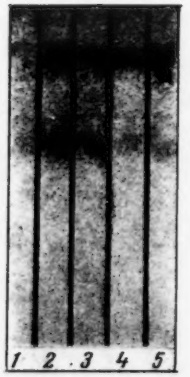

Fig. 3

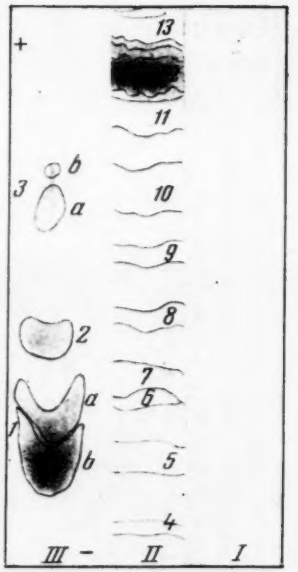

Fig. 4

Fig. 1. Chromatogram of carbohydrates of the acetone-soluble fraction: 1) glucose; 2) acid hydrolyzate of products of alkaline saponification: a) arabinose, b) glucose, c) disaccharide, 3) arabinose; 4) glycerol.

Fig. 2. Chromatogram of the fatty acids, developed with bismuth: 1) oleic acid; 2) standard mixture: stearic, oleic, palmitic acids, 3,4,5,6) lipid fractions: acetone soluble, acetone insoluble, chloroform soluble, bound lipids.

Fig. 3. Chromatogram of fatty acids, developed with potassium permanganate: 1) oleic acid; 2,3,4,5) lipid fractions: acetone soluble, acetone insoluble, chloroform soluble, bound lipids.

Fig. 4. Acetone-soluble fraction. I) Chromatogram of amino acids before hydrolysis and after dialysis; II) chromatogram of amino acids after hydrolysis: 4) valine; 5) tyrosine; 6) proline; 7) alanine; 8) glutamic acid; 9) threonine; 10) glycine; 11) serine; 12) diamino acids; 13) cystine. III) Electrophoretogram after single phase chromatography: 1) diamino acids: a) histidine; 2) arginine; 2) monoaminomonocarboxylic acids; 3) dicarboxylic acids: a) glutamic acid; b) aspartic acid.

A strip of chromatographic paper with dimensions 34 × 2 cm was soaked with 10% solution of paraffin oil in benzene, for which the strip was placed in the chromatograph vessel as if for an ascending chromatogram. After the strip had been air-dried the mixture of fatty acids to be studied was deposited on it in the amount of 50-100 μg. In each chromatographic vessel we placed a control, a mixture of standard fatty acids.

The development of the mixture of fatty acids placed on the paper was carried out with glacial acetic acid by the ascending process. After development the chromatogram was air-dried and washed once with water. Then the fatty acid spots were brought out with a 1% solution of bismuth nitrate and ammonium sulfide. A detailed description of the development of the chromatogram has been given earlier [5].

TABLE 2

Amino Acid Composition of Lipid Fractions After Hydrolysis

Amino acid	Fraction			
	Acetone soluble	Acetone insoluble	Chloroform soluble	Bound lipids
Leucine		one spot	two spot	two spot
Isoleucine	+	++	+++	+++
Phenylalanine			+++	+++
Valine	–	+	+++	+
Tyrosine	+++	–	++	+
Proline	+++	+	+++	+++
Alanine	+++	+++	+++	+++
Glutamic acid	+++	+++	+++	+++
Threonine	+++	++	+++	++
Glycine	+++	one spot	+++	+++
Aspartic acid	+	++	+++	+++
Serine	++	++	++	++
Diaminoacids	+++	+++	+++	+++
Cystine	++	++	++	++

Notes: + very faint ; ++ faint ; +++ clear

For the identification of the unsaturated fatty acids, the paper strip, after development by acetic acid, was treated with a 1% solution of $KMnO_4$ for 15 min, after which the chromatogram was repeatedly washed with water to remove excess reagent from the paper. At the point to which the unsaturated acids had come there was a yellow-brown spot. Saturated acids were not colored by this treatment.

Using the above-described methods, we were able to detect in all the fractions studied stearic, oleic, and one other unsaturated acid which followed closely the front of the solvent. According to the distribution of the spots, this acid could correspond to $C_{10} - C_{12}$, but the absence of a corresponding standard acid does not allow us to make a more exact identification (Figs. 2, 3).

Thus, using the chromatographic method, we have not found any difference in the fatty acid composition of the brucella lipid fractions. It is possible that this is connected with the fact that in natural lipids there is a very complex mixture of fatty acids, difficult to separate.

Study of the Amino Acid Composition. The qualitative amino acid composition in all the fractions, free and bound, was studied before and after hydrolysis by single-phase paper chromatography in a mixture of butanol-acetic acid-water (40:10:50) with later coloration by ninhydrin.

In the acetone-insoluble and chloroform-soluble fractions we observed a considerable amount of diamino acids and some small amount of cystine. In the acetone-soluble fraction and in the fraction of bound lipids, free amino acids were not found. Then all the fractions were submitted to 24 hour hydrolysis by 6 N HCl. The hydrolyzate, freed from humin and HCl was again studied chromatographically. The amino acids found in the different fractions of the lipids are given in Table 2.

For more complete separation of the complex mixture and identification of the diamino acids whose concentration, to judge by the intensity of the color of the spots, was considerably higher than that of the other amino acids, we used the method of ionophoresis in combination with chromatography [6].

The use of ionophoresis permitted us to detect in almost all the fractions three basic amino acids: arginine, lysine, and histidine, except for the acetone-soluble fraction, in which we found only two diamino acids (histidine and arginine). Also, using this method, we obtained a sharper separation of the monoamino dicarboxylic acids – glutamic and aspartic.

As the data in Table 2 show, all the lipid fractions, free and bound, contained 13-15 amino acids. The fact that large amounts of amino acids were detected only after prolonged acid hydrolysis shows the presence in the studied material of high molecular-weight polypeptides or proteins, closely bound to the lipids. This agrees fully with the literature data [7] on the existence of stable lipoprotein complexes.

In order to show the existence of a comparatively strong bound between the lipids and the polypeptides (proteins) we tried experiments with dialysis. A water suspension of the acetone-insoluble fraction was dialyzed for 36 hours against running distilled water. The acetone-insoluble fraction was chosen as the fraction which contained free amino acids before hydrolysis. After the dialysis we did not find any spots on the chromatogram which were revealed by ninhydrin (Fig. 4). This showed that in this material there was no polypeptide or protein not bound to the lipid, but the free amino acids detected before hydrolysis were removed by dialysis.

After hydrolysis with 6 N HCl for 24 hours we again found a large quantity of amino acids. Hence there can be no doubt of the presence of a bond between lipids and peptides (proteins).

When we studied the free and bound lipid fractions by the method of chromatography we discovered in the bound, and even in the free, lipids the presence of components unusual for lipids: carbohydrates and polypeptides or proteins. The presence of these substances in the lipids confirms the possibility of the existence of complex lipid compounds with carbohydates, polypeptides, or proteins.

SUMMARY

We have studied free and bound fractions of lipids obtained from Br. suis 44. The method of paper chromatography shows the presence in all lipid fractions of carbohydrates and polypeptides or proteins.

The data obtained in the study of the products of a single alkaline hydrolysis, or this combined with an acid hydrolysis, permits us to assume that the carbohydrate which is found in the analyzed lipids is a polysaccharide. Before the alkaline hydrolysis, carbohydrate is not found in the studied lipids.

In all the lipid fractions we find glycerol which probably occurs in the lipid composition and splits from it in the alkaline hydrolysis.

The chromatographic method shows in all the fractions the presence of stearic, oleic, and a $C_{10} - C_{12}$ unsaturated acid.

The acetone-insoluble and chloroform-soluble fractions contain free diamino acids and cystine which can be separated by dialysis. None of the fractions show free peptides. After hydrolysis by 6 N HCl we find 13-15 amino acids in the free and bound fractions.

The presence of carbohydrates and polypeptides (proteins) in the lipids confirms the possibility of the existence of complex compounds of lipids with other substances.

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SOME CHARACTERISTICS OF THE PROTEASE OF ESCHERICHIA COLI CULTURED UNDER STATIONARY CONDITIONS AND WITH AERATION

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In a study of the effect of aeration on the growth of E. coli it was established that under these conditions of culturing the total yield of the microbe mass was considerably increased.

The data obtained by Roberts [1] showed that the yield of aerated cultures of E. coli was 34 times higher than the yield of bacteria obtained under ordinary culturing conditions. Such a hastening of the growth of the bacteria by aeration was closely connected with an increased activity of the enzymes of protein metabolism. In this connection it is important to observe that there are especially few data in the literature on the study of the activity of the proteolytic enzymes of E. coli. Bergman and Rettger [2] have shown that E. coli does not excrete proteolytic enzymes into the culture medium. Simmonds and other authors [3] studied the intracellular peptidases of E. coli. Voinar [4] established that on hydrolysis of L-alanylglycine by peptidase, Mg^{++} was required. Green and Neurath [5] showed that Co^{++}, Mn^{++}, Ca^{++}, and Cd^{++} activated trypsin. Jones and a number of authors [6] showed the inhibition of the protease activity of staphylococcus by reducing agents. According to Melchior [7] in the growth of intestinal bacteria, 2, 4-dinitrophenol (DNP) caused a 95% decrease in the biosynthesis of proteins. Sodium azide under conditions of aeration fully blocked the growth of cultures and the biosynthesis of proteins by E. coli.

The purpose of the present work was to study the activity of the protease of E. coli when aerated and under stationary conditions of cultivation.

METHODS

We used E. coli strain 844 for these experiments. After the culture of E. coli was innoculated into agar and meat peptone broth, it was transferred to a microfermenter with Hottinger broth at pH 7.4. The culturing of E. coli was carried out at 37° for 24 hours, at the same time under aeration and stationary conditions. Samples for the study of protease activity were taken after 4, 8, 12, 18 and 24 hour growth of E. coli.

It should be noted that the periods of taking the samples in all cultures of intestinal bacteria agree with the typical curve of the rate of growth of E. coli according to Monod [8], corresponding to the following phases of growth:

1) 4 hours – end of latent phase of growth
2) 8 hours – end of logarithmic phase of growth
3) 12 hours – end of stationary phase of growth
4) 18 and 24 hours – phase of dying bacteria.

In all the samples the microbe mass was separated from the culture medium by centrifuging in the cold, washing three times with physiological saline, and after being ground with powdered glass for 15 min, it was used for the preparation of a 10% bacterial suspension in phosphate buffers at pH 5.0, 6.0, 7.0, 8.0, and 8.3.

In the experimental and control samples, 0.5 ml of toluene was taken for 9 ml of the 10% bacterial suspensions at the various pH values. The experimental samples were incubated for 24 hours at 37°. In the control samples we determined the initial content of basic nitrogen before incubation.

When we studied the effect of foreign proteins on the activity of E. coli protease, the composition of the experimental and control samples was as follows: 8 ml of 10% bacterial suspension prepared in a phosphate buffer with pH 8.0, 1 ml of a 2% solution of the corresponding protein, and 0.5 ml of toluene. Incubation was carried out for 15 hours at 37°. In the control samples we determined the content of basic nitrogen before incubation.

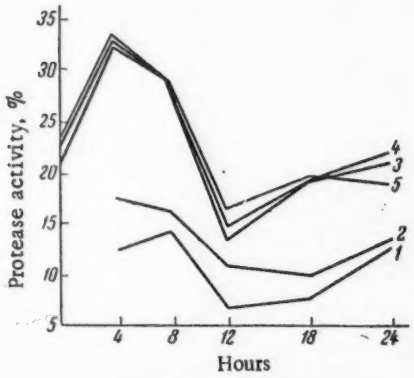

Fig. 1. Change in E. coli protease activity depending on age, with culturing under conditions of aeration.
1) at pH 5.0; 2) at pH 6.0; 3) at pH 7.0; 4) at pH 8.0; 5) at pH 8.3

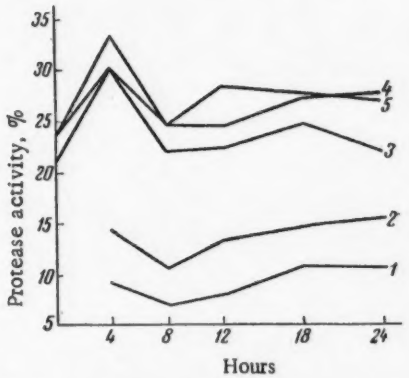

Fig. 2. Change in E. coli protease activity in the dynamics of growth with stationary culturing.
1) at pH 5.0; 2) at pH 6.0; 3) at pH 7.0; 4) at pH 8.0; 5) at pH 8.3

The protein was removed with enough 20% CCl_3COOH so that the final concentration of acid was 5%. The mixture was centrifuged at 6000 r.p.m. In the supernatant liquid we determined the basic nitrogen by micro-Kjeldahl. The activity of the protease was indicated by the rise in the amount of basic nitrogen in the bacterial suspension after incubation as compared to the initial content of basic nitrogen (control experiment before incubation). The content of basic nitrogen in the control samples before incubation was taken as 100.

TABLE 1

Protease Action of Aerated and Stationary Cultures of E. coli On Foreign Protein

Substrate	Aerated culture, %	Stationary culture, %
Control	100	
Casein	+62	+33
Phaseolin	+5	−14
Albumin	+6	−

The activity of E. coli protease was expressed in percent of the control in absolute dry weight of substances. The experimental results shown in Figs. 1, 2, 3, 4, and 5 and in Table 1 are the averages of five experiments.

RESULTS

From the experimental data presented in Figs. 1 and 2 it is evident that the activity of the proteolytic enzymes of E. coli changes with the age of the culture as follows: in the latent and logarithmic phases the proteolytic activity of E. coli increases; in the stationary phase it decreases; and in the final phases of culturing E. coli the protease activity during aeration is lower than in the stationary condition of culturing.

It is also important to notice that during the aeration, the proteolytic activity of E. coli in the range pH 7-8.3 is 1.5-2.5 times higher than at pH 5.0 (Fig. 1). In the stationary culture the protease activity in this range is 2.5-3.5 times higher than at pH 5.0 (Fig. 2). Hence the pH range 7.0-8.3 is the most favorable for the appearance of high proteolytic activity of the E. coli enzymes.

The effect of the conditions of culturing E. coli on the specificity of the intestinal bacteria protease was studied in experiments with the addition to the bacterial suspension of the following foreign proteins: casein, albumin (crystalline), and phaseolin.

The results of these experiments are given in Table 1.

From the data presented in Fig. 1 it is evident that the hydrolysis of casein by the aerated culture of E. coli occurs twice as fast as by the same enzymes of the stationary culture. The hydrolysis of phaseolin and albumin by the proteolytic enzymes of the aerated E. coli culture is 10 times weaker than for casein. It is also important to observe that under these conditions phaseolin causes a 14% decrease in proteolysis by the stationary culture, compared to a 5% decrease for the same protein by the proteases of an aerated E. coli culture (5%) and this gives us reason to suppose that there is the possibility of inactivation of the proteolytic activity of the enzymes of stationary E. coli by phaseolin.

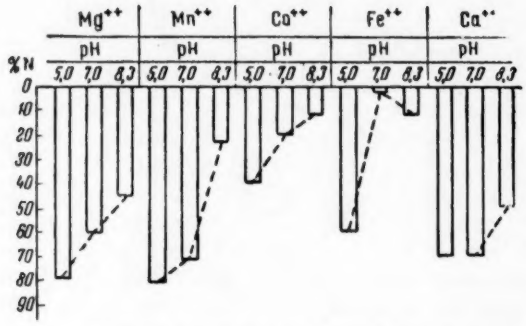

Fig. 3. Effect of metal ions on the activity of proteolytic enzymes of E. coli.
The numbers on the ordinate are the decrease in activity in % N.

Table 2

Concentration of Chemical Compounds Which Produce the Greatest Change in Activity of the Proteolytic Enzymes of E. coli

Chemical compound	Concentration in experimental sample, mM
$MgSO_4 \cdot 7H_2O$	9,0
$MnCl_2 \cdot 4H_2O$	18,0
$CoCl_2 \cdot 6H_2O$	17,0
$CaCl_2$	20,0
$FeSO_4 \cdot 7H_2O$	3,2
Cysteine·HCl	1,8
Methionine	1,5
Thiolactic acid	2,3
$CH_2I \cdot COOH$	1,0
8-Hydroxyquinoline	0,44
NaN_3	10,0
2,4-Dinitrophenol	1,0

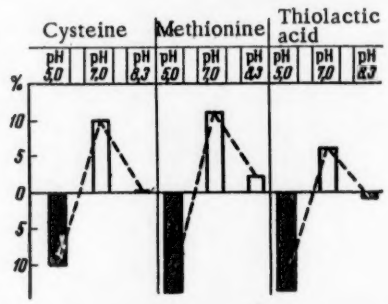

Fig. 4. Effect of S and SH compounds on the activity of proteolytic enzymes of E. coli.

It is known that some chemical compounds such as ions of metals, S and SH compounds, and a number of other substances can affect the activity of proteolytic enzymes. In this connection we have also carried out studies on the change of activity of E. coli under the influence of the following substances: metal ions, S and SH compounds, CH_2ICOOH, NaN_3, 2, 4-dinitrophenol, and 8-hydroxyquinoline.

In these experiments we used 6-8 hour cultures of E. coli.

We made a number of preliminary experiments on the concentrations which produced the greatest change in activity of the proteolytic enzymes of E. coli; the results, given in Table 2, were used in further studies whose results in turn are given in Figs. 3, 4, and 5.

The experimental data on the effect of ions of metals on the activity of the enzymes of intestinal bacteria are given in Fig. 3.

From the data it is evident that Mg^{++}, Mn^{++}, Co^{++}, Fe^{++}, and Ca^{++} inhibit the proteolytic activity of intestinal bacteria.

The inhibiting action of metal ions decreases as the pH is changed from 5.0 to 8.3. Thus, Mg^{++}, Mn^{++}, and Ca^{++} sharply decrease the E. coli protease activity at pH 5.0, 7.0, and 8.3.

Fe^{++} ions at pH 7.0 have a weak inhibiting action on the activity of the enzyme.

The experimental data given in Fig. 4 show that cysteine, methionine, and thiolactic acid have almost the same effect on the proteolytic activity of E. coli enzymes, and at pH 7.0 the protease activity is increased while at pH 5.0 it is inhibited.

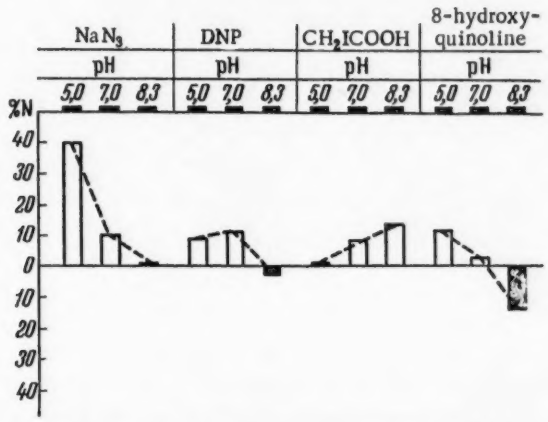

Fig. 5. Effect of NaN_3, dinitrophenol (DNP), CH_2ICOOH, and 8-hydroxyquinoline on the activity of protease of E. coli.

It is interesting to observe the similarity of the effects of methionine and cysteine on the E. coli protease, which can indicate the possibility of conversion of methionine into homocysteine. The experimental data on the effect of some chemical compounds on the activity of the proteolytic enzymes of E. coli given in Fig. 5 shows the following: 1) sodium azide at pH 5.0 raises proteolysis by 38%; 2) 2, 4-dinitrophenol strengthens the proteolytic activity of E. coli in neutral and weakly acid solutions; 3) monoiodoacetic acid at pH 7.0 and 8.3 does not cause change in enzyme activity; 4) 8-hydroxyquinoline at a concentration of $4.4 \cdot 10^{-4}$ M at pH 8.3 blocks the protease activity of E. coli.

In this connection it is also interesting to observe that the direction of change in activity of the proteolytic enzymes under stationary conditions of culturing E. coli was almost the same as in aeration.

SUMMARY

The proteases of E. coli resemble cathepsin in their characteristics as shown by their change in activity at different pH, effect of SH compounds and their relation to monoiodoacetic acid.

The highest proteolytic activity of E. coli is found at pH 7-8.3.

SH compounds at pH 5.0 block the proteases of E. coli and at pH 7.0 raise its activity.

Monoiodoacetic acid does not change the activity of E. coli proteases.

It has been shown that proteases of aerated E. coli cultures hydrolyze foreign proteins more rapidly than do the enzymes of stationary cultures.

8-Hydroxyquinoline blocks the proteolytic activity of intestinal bacteria. Mg^{++}, Mn^{++}, Co^{++}, and Fe^{++} ions decrease the activity of the proteases in aerated and stationary cultures of E. coli.

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A STUDY OF THE REACTIONS OF GRAIN PROTEINS WITH CARBOHYDRATES

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The change in color of food products is usually explained by the formation of melanoids. Melanoids are the dark-colored substances which are formed by the reaction of compounds which contain a primary amino group with compounds which contain a carbonyl group.

We were interested in whether the reaction of melanoid formation could occur in the hydrothermal treatment of grain.

As was shown by the work of Kuzin and Polyakova [1] and Lea and Hannan [2], in the first stages of the reaction between amino groups and carbonyl groups colorless compounds are formed. Only further, deeper transformations of these compounds are accompanied by color changes.

Kretovich and Tokareva [3], Markh [4], and Mashkovtsev [5], as well as other investigators have studied melanoid formation in model substances, prepared from different amino acids and reducing sugars.

Mashkovtsev [5] established that when glucose is heated with glycine without addition of moisture there is an appreciable loss of dry substances.

In order to determine the possibility of melanoid formation in the hydrothermal treatment of grains we carried out experiments with grain proteins and carbohydrates under conditions close to those of the treatment. As objects of study we used the albumin of wheat germ, gliadin of wheat flour, starch, sucrose, and glucose.

For comparison, analogous experiments were carried out with glycine. Proteins, glycine, protein-carbohydrate, and glycine-carbohydrate mixtures were heated in an autoclave under different conditions of temperature and time.

For the determination of the effect of moisture on the process of melanoid formation, we dried part of the substances to constant weight in a vacuum desiccator and heated them without addition of moisture at temperature close to the temperature regime of the autoclave.

In these mixtures we determined the content of dry substances as a result of heating, and also the change in amount of total nitrogen and ammonia in the acid hydrolyzate of heated and unheated samples.

Since acid hydrolysis of heated and unheated protein-carbohydrate mixtures does not exclude the possibility of further reaction between the compounds which are formed in the hydrolysis (according to the data of some authors [6, 7], such reaction can occur at very low pH values), the hydrolysis was carried out in the presence of stannous chloride [8].

EXPERIMENTAL

Wheat protein and starch were isolated as in the previous work [9]. All the starting substances (albumin, gliadin, glycine, starch, sucrose, and glucose) were dried at room temperature in a vacuum desiccator over phosphoric anhydride to constant weight. Thus, dried substances were considered absolutely dry.

In the protein substances we determined the content of total nitrogen, by the micro-Kjehldahl method, and the carbohydrates. The determination of the sugars was carried out by the orcinol method [10]. The results were calculated as glucose. In the albumin we found 14.15% total nitrogen and 25% carbohydrate, in the gliadin, 17.22% total nitrogen and 0.65% carbohydrate, based on the starting weight of the protein sample. All the results are given in percent of starting weight. The nature of the carbohydrate was not established.

TABLE 1

Change in Weight of Samples Heated in an Autoclave in % of Starting Weight

Substance studied	Conditions of heating		Substance studied	Conditions of heating	
	1 atm/hr	2 atm/hr		1 atm/hr	2 atm/hr
Albumin	—2,19	+ 0,23	Glycine and sucrose	—1,84	—20,90
» and glucose	—11,65	—13,07	» » starch	—2,57	+2,73
» » sucrose	—2,87	—12,28	Gliadin	—2,09	+1,71
» » starch	—7,14	— 5,54	» and glucose	+0,27	—16,66
Glycine	+0,64	+ 2,19	» » sucrose	+3,66	+ 2,03
» and glucose	—24,62	—24,14	» » starch	—0,49	+ 2,98

*The sign — means a decrease, the sign + an increase, in weight of the heated sample.

The protein-carbohydrate and glycine-carbohydrate mixtures were prepared with different starting weights of the substances. To guarantee homogeneity we carefully ground the resulting mixtures in an agate mortar, after which they were divided into portions.

The greater part of the absolutely dry, carefully weighted substance was heated in an autoclave in an open test tube under different conditions of temperature and time.

A small amount of the same studied mixtures, dried in a vacuum desiccator over phosphoric anhydride, was sealed in a glass ampule and heated for 1 hour at 131°, which corresponded to the temperature regime in the autoclave at two technical atmospheres (ta).

The samples which had been heated in the autoclave were again dried in the vacuum desiccator to constant weight. The difference in weight before and after heating was expressed in percent of the starting weight (calculated on the absolutely dry substance).

As the results shown in Table 1 make clear, heating the protein substances in the presence of glucose causes a considerable loss in dry substance. For the mixture albumin-glucose the loss in dry substance is even found after heating in the autoclave at 1 ta for 1 hour; for the mixture gliadin-glucose to produce such a loss it is necessary to heat at 2 ta for 1 hour. For both gliadin and albumin, the loss when heated with glucose is less than the loss of dry substance when the mixture glycine-glucose is heated.

Decrease in weight when heating with sucrose is found at higher temperatures than when heating with glucose.

To carry out the acid hydrolysis of heated and unheated protein-carbohydrate mixtures and starting proteins, we placed a 15-20 mg sample of the studied substances in a glass ampule with 20% (6 N) HCl calculated at 1 ml for each 10 ml of sample, and 200 mg of crystalline stannous chloride, after which the ampule was sealed. Hydrolysis was carried out in an autoclave at 1 ta for 12 hours. The weight of stannous chloride (200 mg) was chosen experimentally. As a result of carrying out a series of experiments on hydrolysis in the presence of different amounts of $SnCl_2 \cdot 2H_2O$ we selected (by external signs) the least amount which gave with heating the minimum of formation of a precipitate and light-yellow color of the solution.

Further increase in the amount of $SnCl_2$ in the mixture did not change the external appearance of the hydrolyzate. At the end of the hydrolysis, the ampule was opened and its contents were quantitatively transferred to a 10 ml calibrated flask where an amount of sodium acetate was added to it equivalent to the amount of acid used for hydrolysis (to decrease the total acidity of the solution), and also we added one drop of isoamyl alcohol (to reduce foaming). Distilled water was added to a total volume of 10 ml.

The resulting solution was filtered from the thin surface film of dark-colored substance which formed during hydrolysis and from the precipitated basic tin salt.

In the filtrate we determined the content of total nitrogen and ammonia. As a control we used a solution of 200 mg of $SnCl_2 \cdot 2H_2O$ in 2 ml of 6 N HCl, heated under the same conditions and treated in the same way.

The effect of the $SnCl_2$ on the content of total nitrogen and the hydrolyzate was shown by carring out parallel experiments of hydrolysis in the presence and absence of $SnCl_2$. In all cases the presence of $SnCl_2$ raised the amount of total nitrogen of the hydrolyzate.

The results are given in Table 2.

TABLE 2

Effect of $SnCl_2$ on Content of Total Nitrogen in the Acid Hydrolyzate of Unheated Samples
Content of Total Nitrogen in % of Sample

Studied substance	In presence of $SnCl_2$	In absence of $SnCl_2$
Albumin	12.14	10.68
Glycine and glucose	10.09	7.12
" " sucrose	11.53	9.57
" " starch	9.46	7.41

The data which characterize the content of total nitrogen in the hydrolyzate of heated and unheated forms (Table 3) indicate that the reaction of proteins with carbohydrates is reflected in the content of total nitrogen of the acid hydrolyzates of the products obtained.

The variation in total nitrogen content is not very great; it does not exceed 2–3% of the weight of the starting sample, but it has an entirely regular character. Our results permit us to conclude that the first step in the reaction of protein substances with carbohydrates is not accompanied by any great loss in dry substances but produces an increased content of total nitrogen in the acid hydrolyzate of the substances studied.

Thus, when albumin is heated with sucrose at one ta for an hour, the amount of total nitrogen in the hydrolyzate rises from 6.39% (unheated sample) to 8.74%. The same effect is found for gliadin mixed with glucose and heated at one and two ta for one hour, and for gliadin mixed with sucrose and heated at two ta. In the same cases, when the proteins are heated in the presence of carbohydrates and there is a considerable decrease in dry substance (Table 1) at first there is a rise in content of total nitrogen of thehydrolyzate. Further heating at higher temperature then causes a decreased nitrogen content. For glycine, the change in content of total nitrogen is found to the greatest extent in the sample "glycine and sucrose."

TABLE 3

Content of Total Nitrogen in Acid Hydrolyzate of Substances Studied in % of Weight Calculated on Absolutely Dry Substance

Substance studied	Unheated substance	Conditions of heating				
		Sealed ampoule, 131°, 1 hour	1 atm, 1 hr	2 atm, 1 hr	3 atm, 5 min	4 atm, 5 min
Albumin	12,14	14,37	11,52	13,30	—	—
» and glucose	5,79	7,63	8,84	7,44	—	—
» » sucrose	6,39	6,96	8,74	7,41	—	—
» » starch	5,84	6,76	5,93	6,54	—	—
Gliadin	17,61	16,17	16,98	17,66	15,39	15,91
» and glucose	9,86	9,74	10,42	10,82	7,90	8,85
» » sucrose	8,78	9,23	9,65	10,16	8,07	8,82
» » starch	9,21	9,80	9,38	8,69	10,29	10,10
Glycine	17,08	17,23	17,85	17,46	18,29	18,07
» and glucose	10,09	9,37	10,12	8,74	9,95	8,32
» » sucrose	11,53	11,42	12,74	9,76	8,29	9,09
» » starch	9,46	9,77	9,13	9,38	8,50	9,88

As to the mixture glucose-glycine, here the reaction goes deeply with heating in a sealed tube even in the absence of moisture (as judged by the intensity of darkening of the heated mixture) and this may be why we do not find general regularities in the change of total nitrogen content in the hydrolyzate.

If we consider the increase in total nitrogen as a sign of reaction between the protein substances and the carbohydrates (melanoid formation) then comparison of the total nitrogen content of unheated samples and samples heated in the absence of moisture in the sealed ampules can show that albumin alone and albumin mixed with glucose under these conditions clearly undergo the reaction of melanoid formation. For gliadin such a reaction under these conditions is not found.

We determined the ammonia of the hydrolyzate in percent of the total nitrogen by the method of Belozerskii and Proskuryakov [11]. The results are given in Table 4.

TABLE 4

Content of Ammonia Nitrogen in the Acid Hydrolysis of the Substances Studied In % of Total Nitrogen

Substance studied	Conditions of heating					
	Not heated	Sealed ampoule, 131°, 1 hour	1 atm, 1 hr	2 atm, 1 hr	3 atm, 5 min	4 atm, 5 min
Albumin	10,40	13,50	8,50	5,02	—	—
» and glucose	8,46	10,78	8,97	9,38	—	—
» » sucrose	7,99	5,98	9,24	7,40	—	—
» » starch	8,27	8,21	8,44	8,87	—	—
Gliadin	18,70	19,70	12,09	24,07	23,22	19,88
» and glucose	22,83	18,12	24,16	30,85	27,39	36,76
» » sucrose	23,25	19,15	14,88	19,23	19,14	20,31
» » starch	18,05	19,11	18,09	25,99	27,77	25,14
Glycine	0,95	0,49	0,98	0,45	—	—
» and glucose	3,85	8,64	6,72	5,75	—	—
» » sucrose	1,38	3,14	5,28	10,97	—	—
» » starch	1,09	1,20	2,68	3,51	—	—

These results permit the assumption that heating the protein mixed with carbohydrate and even hydrolysis of the protein in the presence of one or another carbohydrate and also an energetic reducing agent like $SnCl_2$ is reflected in the ammonia content in the hydrolyzate. At the same time, comparison of all the analytical results shows that general regularities for all the mixtures cannot be established here.

When glycine and albumin are heated with different carbohydrates under definite heating conditions there occurs some maximum in the ammonia content and the quantity falls with further heating. When gliadin is heated under definite conditions mixed with glucose or sucrose there is a decreased content of ammonia in the hydrolyzate with later increase in its amount when the conditions of heating are changed.

We can assume that different formation of ammonia in the hydrolysis of heated protein-carbohydrate mixture is connected with the nature of those amino acids which under various conditions of heating react with the carbohydrates in the mixture.

SUMMARY

Heating the proteins of wheat grains (albumin and gliadin) mixed with carbohydrates (glucose, sucrose, and starch) under the conditions of hydrothermal treatment is accompanied in a number of cases by reaction of the proteins with the carbohydrates.

The depth of the reaction depends on the nature of the proteins and the character of the carbohydrates with which the proteins are heated.

The first sign of such a reaction is the change (increase) in content of total nitrogen in the acid hydrolyzate of the substances studied.

The depth of the reaction between proteins and carbohydrates can be characterized by the considerable loss of dry substance of the heated mixture.

The reaction of proteins with carbohydrates is accompanied by a change in ammonia content in the acid hydrolyzate of the substances studied.

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THE SEPARATION OF ADENOSINE PHOSPHORIC ACIDS ON DOMESTIC ANIONITES

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The study of nucleotide metabolism requires the development of methods which permit quantitative separation of mixtures of nucleotides. In recent years, anion exchange resins have been widely used abroad for this purpose; the most used has been Dowex-1. Domestic anionites have been little studied from this point of view.

We have set ourselves the problem of choosing conditions for the separation of the more complex mixtures of nucleotides; we have begun to work with a number of strongly basic domestic anionites, investigating their ability to separate mixtures of adenosine phosphoric acids of known composition. The separation of adenosine phosphates (and inosine phosphates) on domestic anionites has been the subject of recently published work of Gorkin [1] but the conditions which he used for nucleotide separation differed considerably from ours.

EXPERIMENTAL

We used for this work the resins: ÉDÉ-10-P *, AV-16, and AV-17.** The first two resins are of the condensation type; resin AV-17 is a polystyrene monofunctional resin with a quarternary ammonium base as the ionogenic group, most closely corresponding to Dowex-1.

The resins were dried, ground in a coffee mill, sifted through a 100-mesh sieve, freed by water from the fine particles which did not settle in 5 min after sedimentation, and transferred to a column, either in the Cl' form, by passing 1 N HCl and then washing with distilled water to pH 4–5, or in the formate form, in this case by passing 85% HCOOH through the resin for AV-17 and 1 N HCOOH for AV-16, and then water.

For analytical purposes we used a column about 3 cm high with a cross section 0.6–0.8 cm^2 and separation of 10 mg of ATP; for preparative purposes the column was 4–8 cm high with a cross section 0.6–1.1 cm^2 and separation of 100–300 mg ATP.

As a source of adenosine phosphoric acids we used a commercial preparation of ATP, prepared by the Ivanov Meat Combine. The ATP preparation was first studied by quantitative paper chromatography and spectrophotometry for ATP, ADP, and AMP in two solvent systems: phosphate-isoamyl alcohol (ascending chromatography) according to Cohn and Carter [2], and isobutyric acid – NH_3 – Versene (descending chromatography) according to Krebs and Hems [3]. As the standard we used a mixture of ATP, ADP, and AMP. The spots which absorbed in the ultraviolet were detected in the ultrachemiscope (guanosine phosphates after treatment of the chromatogram with HCl vapors according to Venkstern and Baev [4]); they were cut out, eluted with 0.01 N HCl and determined by extinction at 260 mμ (E_{260}), subtracting from it the value for the difference in size of the control.

In Table 1 we give the comparative results of the determination of adenosine phosphoric acids by these methods for one of the ATP preparations obtained in 1955. The content of AMP, ADP, and GP (guanosine phosphates) differed in the determinations by the two methods within the limits of 3%. The ATP content was several times lower in the system isobutycic acid–NH_3– versene (61%) than in the system phosphate–isoamyl alcohol (67%); this is apparently due to the fact that in the second system, adenosine polyphosphates are not separated from ATP.

* We thank scientific worker of the Institute of Biological and Medical Chemistry, AMN, USSR, V. Z. Gorkin for giving us samples of the resin ÉDÉ-10-P, prepared in the Leningrad Plastics Factory.

** We express deep thanks to scientific worker of the Plastics Research Institute (Ministry of Chemical Industry, Moscow) A. B. Pashkov for preparing the experimental samples of resin AV-17.

TABLE 1

Content of ATP, ADP, AMP in Commercial Preparation of ATP as Determined by Paper Chromatography

System	AMP		ADP		ATP		APP**		GP	
	R_f and R_{AMP}*	% E_{260}	R_f and R_{AMP}	% E_{260}	R_f and R_{AMP}	% E_{260}	R_f and R_{AMP}	% E_{260}	R_f and R_{AMP}	% E_{260}
Phosphate-isoamyl alcohol (ascending chromatogram	0,72	5,5	0,80	21,4	0,84	67,0	—	—	0,89	5,7
Isobutyric acid-NH_3-versene (descending chromatogram	1,00	3,0	0,85	24,0	0,73	61,0	0,60	8,2	0,49	3,5

* R_{AMP} relates to the descending chromatogram.

** Unidentified spot in the system isobutyric acid–NH_3–versene, which corresponds to the supposed adenosine polyphosphates (APP).

For separation on the column, the ATP was dissolved in water, neutralized with 1 N NH_4OH to pH 6.8, water was added to a volume of 5–10 ml, and it was added to the column, passing slowly through the layer of resin. The liquid which passed out the bottom was collected for determination of the completeness of absorption; the column was washed with water until E_{260} did not fall to zero. In part of the ATP solution, diluted 100 or more times with 0.01 N HCl, we determined the starting E_{260}. Because of nonabsorption and washing with water a small portion of nucleotides was removed (usually less than 1% E_{260}).

For elution, the column was joined by a glass tube to a reservoir placed at a height of 1 meter over the column to supply hydrostatic pressure. Elution of the nucleotides was carried out with HCl concentrations not above 0.01 N (in the case of the Cl' form of the resin) so as to avoid hydrolysis of the labile nucleotides. A fraction with a volume of 10–15 ml was collected with an automatic collector; the rate of elution was set at about 1 ml per min. When the column remained over night, the rate of elution at the end of passing the solution had slowed somewhat because of the fall in hydrostatic pressure and the volume of the fraction decreased; therefore the volume was always measured. As a basis for the separation of adenosine phosphates on the resin in the Cl' form we took the method of Cohn and Carter [2] which was worked out for Dowex-1, and which we modified depending on the nature of the resin.

In Figs. 1–3 we give diagrams of the separation of adenosine phosphates in ATP preparations for resins EDE-10-P, AV-16, and AV-17. On the abscissa we have laid out the volume of the solutions with which elution was produced and on the ordinate on the one hand E_{260} of the fractions, and on the other, the pH change in them.

On Resin ÉDÉ-10-P (Fig. 1) AMP was eluted by 0.01 NaCl in 0.005 N HCl, ADP by 0.04 N NaCl in 0.01 N HCl, and ATP by 0.5 N NaCl in 0.01 N HCl. For resin AV-16 we used the solutions employed by Cohn and Carter, that is, AMP was eluted by 0.003 N HCl, ADP by 0.02 N NaCl in 0.01 N HCl, and ATP by 0.2 N NaCl in 0.01 N HCl. For resin AV-17 the most suitable solutions were those which eluted ADP and ATP at even lower NaCl concentrations than in the Cohn-Carter method: AMP, 0.005 N HCl; ADP, 0.01 N NaCl in 0.01 N HCl; ATP, 0.1 N NaCl in 0.01 N HCl. For all the resins which we studied, under the conditions used the elution was almost quantitative (see figs.); more than 95% of the initial E_{260} was eluted. In all cases, working with the Cl' form of the resin, elution was completed with 1 N HCl, and resin AV-17 was also washed with concentrated HCl to remove nucleotide residues.

As the figures show, all three resins under these conditions of elution separate the adenosine phosphoric acids quantitatively: in the first fraction (AMP) we found 6–7% of the nucleotides, in the second (ADP) 20–24%, and in the third (ATP) 60–67%, which agrees with the data of Table 1.

Resins of the condensation type, ÉDÉ-10-P and AV-16, have a definite buffering action. In Figs. 1 and 2 we see that in the elution by the first solution a rather large amount of liquid is passed before the pH of the passing li-

quid reaches the pH of the solution used for evolution, and only after this does the elution of adenylic acid begin. Resin AV-17 has almost no buffer action, since it is a more strongly basic resin, with a quaternary ammonium bas as its monofunctional group.

TABLE 2

Preparative Separation of a Commercial Preparation of ATP on Resins AV-16 and AV-17

Resin	Dimensions of resin column	ATP used, mg	Found E_{260}, %				Total yield in % E_{260}
			AMP	ADP	ATP	Nucleotides eluted by 1 N HCl	
AV-16	4 × 0.6 cm^2	100*	6	23	60	6.5	96
AV-17 × 10**	8 × 1.1 cm^2	300	9	28	51	8	95.5

* According to the results of paper chromatography, the preparation contains 7-8% AMP, 22-23% ADP, and 69-70% ATP.

** × 10 = 10% divinylbenzene (cross section).

Preparative separation of a commercial preparation of ATP was carried out on resins AV-16 and AV-17 (Cl' form) under the same conditions of elution as in the work with the analytical column, but for resin AV-17 elution with 0.2 N NaCl in 0.01 N HCl was replaced by elution with 1 N HCl. The nucleotides were eluted as four separate peaks, as in the fractionation of ATP for analytical purposes. In Table 2 we give the results of two similar experiments.

In these experiments we used different preparations of commercial ATP, differing in the time of obtaining and of keeping from the ATP preparations shown in Table 1. To judge the purity of the nucleotide fractions which we obtained, we isolated them in the preparative method as their Ba salts and tested their homogeneity by paper chromatography. For the preparative separation we combined the fractions with high nucleotide concentrations and rejected the marginal fractions.

For resin AV-16, from which the nucleotide fractions were eluted with a rather large volume of solution (500-1200 ml) the ADP and ATP fractions after neutralization were treated with activated charcoal, type OU GOST 4453-48, to absorb the nucleotides, which were eluted from the charcoal with 40% alcohol made alkaline with ammonia according to Cabib, Leloir, and Cardini [5]. The yield after elution from charcoal was about 80%. The extracts were concentrated by vacuum distillation and precipitated as the Ba salts in the presence of 50% alcohol. AMP was obtained directly by concentration of the solution in a vacuum.

For resin AV-17, from which even the ATP fraction was removed by NaCl solutions with concentrations not above 0.1 N NaCl, it was possible to obtain pure preparations of ADP and ATP without absorption on charcoal, which reduced considerably the loss in the preparative isolation. After separation on the column, the ADP and ATP fractions were quickly neutralized. The ADP fraction, which contained 0.02 N NaCl, was evaporated in a vacuum to about 1/10 its volume and precipitated by barium acetate in the presence of 50% alcohol; the ATP fraction was reduce to 1/5-1/6 its volume and precipitated by the same process. From 300 mg of free ATP we obtained 90 mg of the Ba salt of ADP and 150 mg of the Ba salt of ATP. Because of the low content of AMP, it was not isolated preparatively in these experiments.

All the resulting fractions of nucleotides were studied by paper chromatography. For the experiments we used Leningrad slow chromatographic filter paper.

Before use, it was washed in 0.1 N HCl and then in distilled water or in 0.5% Versene, pH 8.5 and again in water. In the preparations of AMP and ADP obtained on a column of AV-16 resin, after chromatography in the system ethyl alcohol-ammonium acetate, pH 3.8, (according to Paladini and Leloir [6]), we found only one spot, that is, both fractions were chromatographically pure. In the ATP fraction, besides the ATP spot, we found a weak spot of ADP which was apparently formed from ATP in the process of fractionation on the column and preparative treatment.

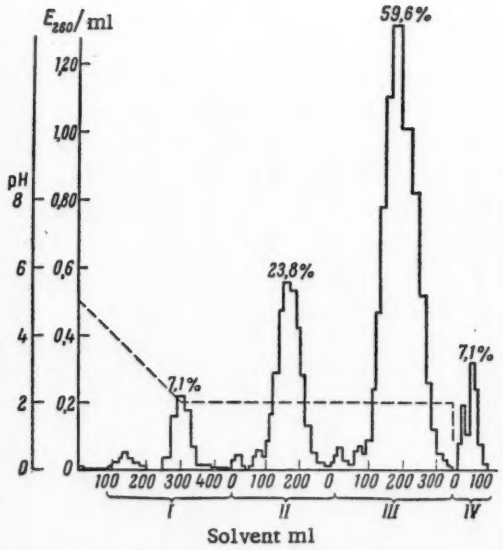

Fig. 1. Separation of adenosine phosphoric acids (10 mg of commercial ATP) on a column with resin EDE-10-P (Cl').
Solid line – E_{260}/ml; dotted line – pH. Figures at the peaks – % extinction at 260 mμ for the given solution. Yield 97.6%. Eluting solutions: I) 0.01 N NaCl in 0.005 N HCl (AMP); II) 0.04 N NaCl in 0.01 N HCl (ADP); III) 0.5 N NaCl in 0.01 N HCl (ATP); IV) N HCl (unidentified nucleotides).

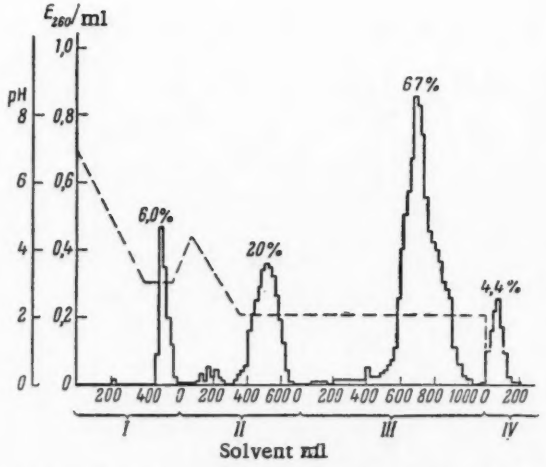

Fig. 2. Separation of adenosine phosphoric acids (10 mg of commercial ATP) on a column with resin AV-16 (Cl').
Designations the same as in Fig. 1. Yield 97.4%. Eluting solutions: I) 0.003 N HCl (AMP); II) 0.02 N NaCl in 0.01 N HCl (ADP); III) 0.2 N NaCl in 0.01 N HCl (ATP); IV) N HCl (unidentified nucleotides).

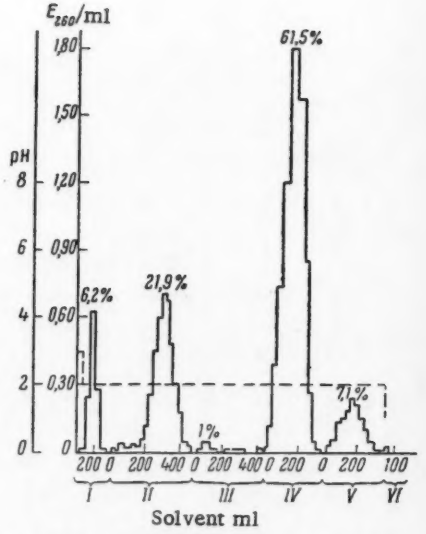

Fig. 3. Separation of adenosine phosphoric acids (10 mg of commercial ATP) on a column with resin AV-17 × 2 (Cl'). Designations the same as in Fig. 1. Yield 97.7%. Eluting solutions: I) 0.005 N HCl (AMP); II) 0.01 N NaCl in 0.01 N HCl (ADP); III) 0.05 N NaCl in 0.01 N HCL; IV) 0.1 N NaCl in 0.01 N HCl (ATP); V) 0.2 N NaCl in 0.1 N HCl (unidentified nucleotides); VI) N HCl.

The preparations of ADP and ATP obtained by fractionation on a column with AV-17 were studied in the system phosphate-isoamyl alcohol and isobutyric acid–NH_3–versene. In both systems, ADP and ATP gave one spot when the chromatograms were viewed under the chemiscope and compared with a standard. However, the chromatogram of pure ATP, after a short period of keeping it, began to show small quantities of ADP.

When 500 mg instead of 300 mg of ATP was fractionated on a column with resin AV-17 (8 ×1.1 cm^2), complete separation of the nucleotides was not attained; in the elution of fractions of AMP (0.005 N HCl) and ADP 0.02 N NaCl in 0.01 N HCl) E_{260} rose to a maximum and then did not fall to zero, but remained at a rather high level before removal of the following fraction. The ATP fraction in this experiment was not fully eluted by 0.1 N NaCl in 0.01 N HCl (40% E_{260} instead of 51%). 16% of the total E_{260} in this case remained on the resin, even when the column was washed with 1 N HCl. Thus, resin AV-17 has appreciably less capacity than the various forms of foreign anionites.

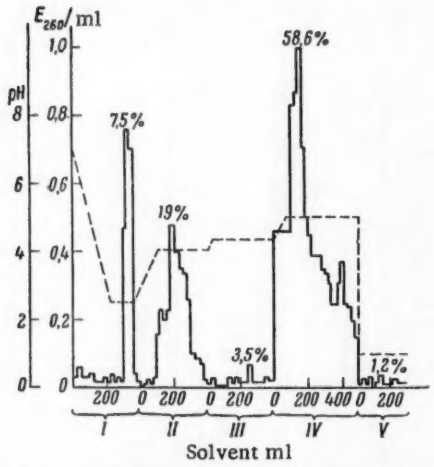

Fig. 4. Separation of adenosine phosphoric acids (10 mg of commercial ATP) on columns with resin AV-16 (formate).
Designations the same as in Fig. 1. Yield 90%. Eluting solutions: I) 0.1 N HCOOH (AMP); II) 0.3 N HCOONa in 0.1 N HCOOH (ADP); III) 0.5 N HCOONa in 0.1 N HCOOH; IV) N HCOONa in 0.1 N HCOOH (ATP); V) N HCOOH.

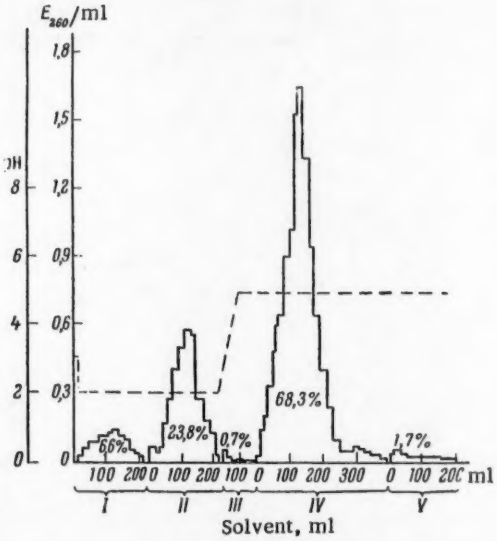

Fig. 5. Separation of adenosine phosphoric acids (10 mg of commercial ATP) on a column with resin AV-17 × 2 (formate).
Designations the same as in Fig. 1. Yield 101%. Eluting solutions: I) 0.05 N HCOOH (AMP); II) 2.5 N HCOOH (ADP); III) 0.4 N $HCOONH_4$; IV) 0.7 N $HCOONH_4$ (ATP); V) 1.25 N $HCOONH_4$.

The separation of ATP preparations for analytical purposes by the formate system was carried out on resins AV-16 and AV-17. For resin AV-16 we used the system formic acid-sodium formate, according to Bergkvist and Deutsch [7], with some changes in the concentrations of the eluting solutions (Fig. 4). AMP was eluted with 0.1 N HCOOH, ADP with 0.3 N HCOONa in 0.1 N HCOOH, and ATP in 1 N HCOONa in 0.1 N HCOOH. Elution was almost quantitative; the total yield based on E_{260} was 90%. The fraction with 1 N HCOONa and 0.1 N HCOOH contained, besides the usual peak which corresponded to ATP (53% E_{260}), a second small peak, corresponding to 5.3% E_{260}, which probably came from the unidentified nucleotides in the ATP preparation.

In resin AV-17 we tested the separation of adenosine phosphates in the formate system: formic acid-ammonium formate, according to Herbert, Potter, and Takagi [8]. In Fig. 5 we give the results of the separation of ATP in this system, carried out with the same preparation of ATP as for the experiments given in Table 1. AMP was washed out with 0.05 N HCOOH, and ADP with 2.5 N HCOOH respectively [8]. ATP was not eluted with 1.25 M ammonium formate, as by these authors [8], but by 0.7 M ammonium formate. The elution was quantitative, the yield by E_{260} was 101%. In this system the unidentified nucleotides apparently were not separated from ATP, since the ATP fraction was somewhat high. We made no attempt in this or the other formate system to separate the unindentified nucleotides from ATP by selecting the conditions of elution.

DISCUSSION OF RESULTS

Our data show that all three studied domestic anionites, ÉDÉ-10-P, AV-16, and AV-17, in the Cl^- form, under definite elution conditions separate quantitatively a mixture of adenosine phosphoric acids. The one most suitable for the separation is resin AV-17 which does not have a buffer action and with which nucleotides are eluted at low concentrations of HCl and NaCl. With resins of the condensation type, ÉDÉ-10-P and AV-16, elution of the nucleotides is more difficult than with AV-17, but in the case of AV-16 we obtained a quantitative elution by using solutions of low concentration according to Cohn and Carter [2], and for ÉDÉ-10-P with concentrations of HCl not surpassing 0.01 N and NaCl concentrations of 0.5 N.

In the work of Gorkin [1] for the separation of analytical amounts of adenosine phosphates, high concentrations of HCl were used: 0.1 N HCl for the elution of ADP, 0.2 N HCl for ATP. Since the rate of elution was very small and the process of separation evidently occupied several days, then it is very likely that in the process of separation on the column, part of the ATP was transformed into ADP and AMP, and part of the ADP into AMP. For preparative separation of adenosine phosphates Gorkin used high concentrations (up to 1 M) of NaCl in 0.01 N HCl for elution. The use in our experiments with resin AV-17 of an eluting solution with low salt concentration permitted us to separate the nucleotides preparatively without adsorption on charcoal. The volume of the fraction was then 250-700 ml.

SUMMARY

We have worked out conditions for quantitative separation of adenosine phosphates on domestic anionites ÉDÉ-10-P, AV-16, and AV-17 in the Cl^- form. The best results were obtained with resin AV-17. Resins AV-16 and AV-17 also separate adenosine phosphates in the formate system.

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THE POLAROGRAPHIC DETERMINATION OF PROTEASES AND AMYLASES OF BACILLUS SUBTILIS

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The polarography of proteins was first carried out by Brdicka [1] who obtained a double wave from reduction of the proteins of blood serum, using an ammonia buffer with $CoCl_2$ as the indifferent electrolyte. A comparative study of different proteins (albumin, globulin fibrinogen, plasma and blood serum) on the polarograph was carried out by Tropp and co-workers [2, 3]. Polarography was also used in studying the denaturation of proteins under the influence of alkalis and acids [3-5] and for the determination of gluten in different types of wheat flour [6].

In the first work on polarography of proteins [1, 2] it was assumed that the double reduction wave (especially the second wave) was related to reduction of the –S–S– bond in the protein molecule. Very recently, however, it has been shown that proteins in which the SH groups are blocked by heavy metals also give the polarographic wave [7]. At the present time it is considered that the double wave occurs as a result of reduction of the hydrogen bonds of different groups in the protein molecule, including also the hydrogen of the SH group.

Besides the proteins which have the double wave there are proteins which show a single reduction wave (for example, the proteins of saliva).

In the present communication we give data on the polarographic behavior of proteases and amylases of B. subtilis. This work shows the need for finding a qualitative method for controlling the purification of culture media.

METHODS AND MATERIALS

In the experiments we used crystalline preparations of protease (13,000 units/g) and amylase (200,000 units/g) obtained from the Japanese firm, Nagase. The crystalline amylase peparation contained a contaminant of protease amounting to 700 units/g of protein (the activity of the protease was determined by titration of an alkali hydrolyzate of casein after precipitation of the protein with hydrochloric acid).

For comparison of the nature of the waves in the experiment we also used a pure preparation of egg albumin which had a double reduction wave. The solutions of the enzymes were prepared from air-dried preparations.

Polarography was carried out on a mirror polarograph SGM-8 from the "Geological Survey" factory.

The height of the mercury column was 65 cm; $m = 1.76$ mg/sec; $S = 1/200$; $T = 3.1$ sec. During polarography the temperature in the liquid medium was 17-18°. The determination was carried out against a saturated calomel electrode.

Samples for polarography contained 0.2–1.8 ml of protease solution (in other experiments, correspondingly, we used 2 ml of amylase solution or 1 ml of egg-albumin solution). All the preparations were used in concentrations of 1 mg/ml; the volume of the protein solution was brought to 2 ml with water, 0.5 ml of agar-agar solution (1 mg/ml) was added, and 2.5 ml of ammonia buffer containing $CoCl_2$ (0.1 M NH_4OH, 0.1 M NH_4Cl, and 0.01 M $CoCl_2$) was also added. In some experiments cobalt was excluded from the buffer mixture. The total volume of the sample was 5 ml.

EXPERIMENTAL

The first question which we asked was, what was the polarographic wave for amylase and protease, and what was its nature. In Fig. 1 we see that amylase on a background of cobalt does not give any apparent reduction wave; protease shows a clearly expressed single wave compared with the double wave of egg albumin.

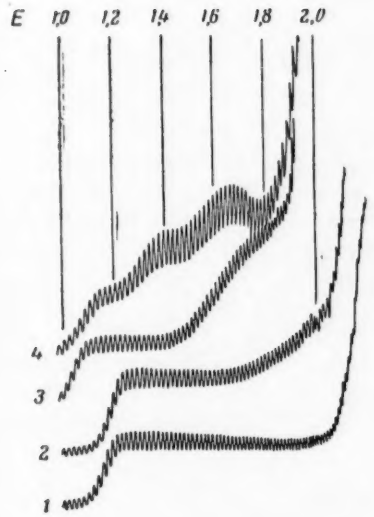

Fig. 1. Comparative polarographic waves of protease and egg albumin: 1) ammonia buffer + $CoCl_2$; 2) amylase 2 ml; 3) protease 1.5 ml; 4) egg albumin 1 ml.

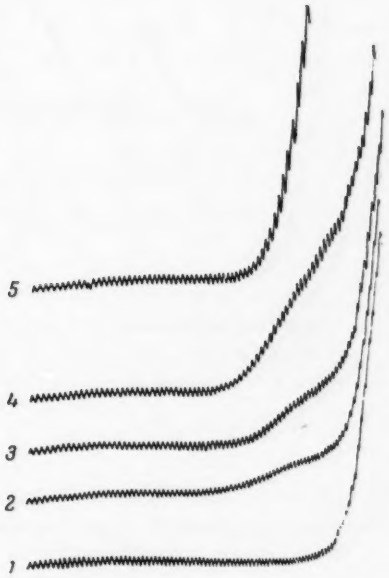

Fig. 2. Significance of cobalt for the occurrence of a polarographic wave: 1) ammonia buffer without cobalt; 2) amylase solution 2 ml; 3) protease solution 0.5 ml; 4) protease solution 2 ml; 5) egg albumin solution 2 ml.

Polarography began at 0.9 V; $E_{\frac{1}{2}}$, wave of protease at 1.59 V. The wave occurred at E = 1.37 V.

In the next series of experiments to discover the role of cobalt in the occurrence of the wave, it was excluded from the composition of the ammonia buffer. In the polarographic curves shown in Fig. 2 we see that in the absence of cobalt the amylase preparation shows a somewhat clearer single wave; protease shows a clear single wave which increases with the protein concentration, and egg albumin does not give a wave at all (Fig. 2). We must remark that the wave which appears in the polarography of amylase may be due to the presence of protease in the amylase preparation. The results show that cobalt affects the amplitude of oscillation of the mirror galvanometer but does not affect the quantitative relation between height of the wave and concentration of protease. Also, the single reduction wave of protease occurs without participation of the cobalt. This differentiates protease from the other proteins which we have studied.

The role of SH groups in the occurrence of the single protein wave becomes doubtful since it has been observed that there is no amino acid with an SH group in the protease of B. subtilis [8].

In Fig. 3 we give the results which show that there is a linear relationship between the protease concentration and the height of the wave (up to 250 mg/ml).

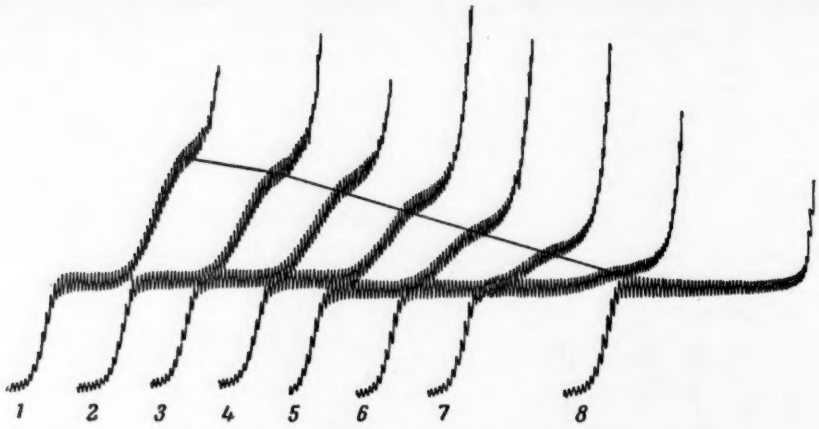

Fig. 3. Quantitative relation between protease concentration and height of wave (h). Protease concentration in μg/ml, height of wave in mm: 1) 280 μg/ml, h = 26 mm; 2) 240 μg/ml, h = 23 mm; 3) 200 μg/ml, h = 20 mm; 4) 160 μg/ml, h = 17 mm; 6) 80 μg/ml, h = 7 mm; 7) 40 μg/ml, h = 2 mm; 8) ammonia buffer + $CoCl_2$.

SUMMARY

We have established that the protease extracted from the culture medium of B. subtilis on a background of ammonia buffer in the presence or absence of cobalt chloride shows single wave reduction at $E_{\frac{1}{2}}$ equal to 1.59 V. There is a quantitative relation between protease content and height of wave. Amylase in the presence of cobalt chloride does not give a notable reduction wave.

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SOME TRANSAMINATION REACTIONS OF AMINO ACIDS IN INTESTINAL BACTERIA

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The synthesis and decomposition of L–amino acids occurs by the transamination reaction [1]. The wide distribution of the transamination reaction in animal tissues, plants, and microorganisms, and also the great speed of this reaction indicate its great importance in nitrogen metabolism.

In the studies of Cammarata and Cohen [2] it was shown that 25 amino acids took part in this reaction.

Using as the enzyme preparation dried cells of Escherichia coli, Pseudomonas fluorescens, Bac. subtilis, Feldman and Gunsalus [3] showed the presence of the reaction of transamination between α-ketoglutaric acid and a large number of amino acids.

In recent years the transamination reaction has been observed in many other studies of microorganisms [4-7].

The present work is devoted to a study of some transamination reactions of amino acids in intestinal bacteria.

PLAN OF EXPERIMENTS AND METHOD OF INVESTIGATION

In this work we used a strain of intestinal bacteria obtained from the Rostov-on-Don Institute of Epidemiology, Microbiology, and Hygiene. The experiments were carried out with extracts of the bacterial cells, since the whole cell was inactive. The bacteria were grown on 5% nutritive agar for 20 hours at 37°. The cultures were twice washed free of medium by physiological saline. The collected bacterial mass was ground in the cold with glass powder and extracted with a phosphate buffer, pH 8.0. After the sediment had been separated by centrifuging, the resulting clear extract was dialyzed against running tap water.

In some experiments we used an acetone preparation of cells. For this the bacterial mass was washed with ten times the amount of cold acetone and ether. Such a preparation at 0° retained its activity for months.

Composition of the control samples: first, 10 μmole of α-ketoglutarate, second, correspondingly, 10 μmole sodium pyruvate, phosphate buffer M/15, pH 8.0, and the bacterial extract. In the experimental samples, besides this, we added 10 μmoles of the corresponding amino acid, neutralized with sodium bicarbonate to bromothymol blue. The samples were incubated two hours at 37°. The protein was precipitated with 96% ethyl alcohol and separated by centrifuging. The centrifugate was evaporated dry on the water bath and the dry residue was dissolved in 1 ml of distilled water. Glutamic acid and alanine, formed in the transamination, were separated by paper chromatography [8].

We placed on the chromatogram about 25 μl of concentrated, deproteinated filtrates of the control and experimental samples, and also a neutralized standard solution of amino acids. Phenol saturated with water was used as the solvent. After 18 hours the chromatogram was removed from the chamber and the excess phenol was extracted with ether. The chromatogram was dried in air and brought out by immersion in a 1% solution of ninhydrin in 96% ethyl alcohol. After two hours the plainly evident color corresponded to the position of the amino acids. The spots of amino acid which formed as a result of transamination were cut from the paper and the color was eluted from the shredded paper with 3 ml of 50% ethyl alcohol for 15 min.

In the photometric determination we used as the blank a piece of the paper on which solvent had been passed. The color was determined photometrically with a yellow filter in the FEK-M photometer.

Calculation of the amount of amino acid formed was carried out from the calibration curve of the standard solutions of glutamic acid and alanine which contained 1 μg of amino acid per μl. We placed on the paper 10, 20, and 30 μg of glutamic acid or alanine.

The activity of the reaction of transamination of the amino acid with α-ketoglutarate was determined by the amount of glutamic acid formed on the chromatogram. The amount of glutamic acid formed on the chromatogram was calculated in μmoles per 1 ml of sample.

The intensity of the transamination reaction of the amino acids with pyruvate was determined by the amount of alanine formed in μmoles per 1 ml of sample.

TABLE

Transamination in Extracts of Intestinal Bacteria

Amino acid	Ketoacids					
	α-Ketoglutarate			Pyruvate		
	Glutamic acid formed, μmoles	Number of experiments	Limits of variation	Alanine formed μmole	Number of experiments	Limits of variation
Aspartic	4,56	6	3,6—5,5	2,1	3	1,8—2,5
Glutamic	—	—	—	2,16	3	2,0—2,3
Valine	3,36	5	2,5—4,4	1,70	3	1,5—2,0
Leucine	3,0	3	2,0—4,4	1,73	3	1,5—2,0
Phenylalanine	2,97	4	2,2—4,4	—	—	—
Alanine	2,36	3	2,2—2,5	—	—	—
Tryptophan	—	—	—	1,55	2	1,3—1,8
Glycine	1,0	3	0,9—1,1	—	—	—

RESULTS OF THE INVESTIGATION

We studied the transamination reaction of α-ketoglutarate with aspartic acid, valine, leucine, phenylalanine, alanine, and glycine, and of pyruvate with glutamic and aspartic acids, valine, tryptophan, and leucine.

As the Table shows, the most intense tranamination reaction occurred between α-ketoglutarate and aspartic acid. The transamination of α-ketoglutarate with monocarboxylic amino acids was less intense. The least active transamination of α-ketoglutaric acid was with glycine, in which only 1.0 μmole of glutamic acid was formed.

Analogous facts were found in the study of transamination of pyruvate and amino acids.

It follows from the Table that transamination of pyruvate occurs most intensely with glutamic and aspartic acids, and more weakly with valine.

Our data show that the transamination reaction of amino acids with α-ketoglutarate proceeds more intensely than the corresponding transamination reaction of amino acids with pyruvate.

Thus, the transamination reaction of amino acids with dicarboxylic substrates is more intense than this reaction with monocarboxylic substrates.

The most intense transamination is between the two dicarboxylic substrates, α-ketoglutarate and aspartic acid. Our data agree with the idea of Braunshtein, Euler, and Schoenheimer of the central role of glutamic (α-ketoglutaric) acid in the reactions of nitrogen metabolism of amino acids.

SUMMARY

In extracts of E. coli there are enzyme systems which carry out transaminations of α-ketoglutarate with the following amino acids: aspartic acid, valine, leucine, phenylalanine, alanine, and glycine. Of these reactions, the most intense is the transamination of α-ketoglutarate with aspartic acid.

We have observed the presence of a transamination reaction of pyruvate with glutamic acid, aspartic acid, valine, leucine, and tryptophan. Of these the most intense is the transamination of pyruvate with the dicarboxylic amino acids.

The transamination reaction with a dicarboxylic substrate, α-ketoglutarate, is more intense than the corresponding reaction with pyruvate.

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A METHOD OF ISOLATION OF TRANSGLUCOSYLASE FROM THE LIVER AND DETERMINATION OF ITS ACTIVITY

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As our investigations have shown, the liver contains an enzyme, transglucosylase of a non-phosphorolytic type, which has the property of catalyzing the formation of polysaccharides in the presence of α-dextrins and free glucose [1, 2].

In the present communication we describe a newly worked-out method for the isolation of this enzyme from liver which permits us to obtain the enzyme as a crystalline preparation and we have also developed a process for determination of the enzyme.

EXPERIMENTAL

Method of Determining the Activity of the Enzyme. The activity of the enzyme was determined by increase in the amount of oligo- and polysaccharides in an incubation mixture which contained the enzyme (3 ml), α-dextrins (1.5 ml)(Table 1), free glucose (40 mg), and acetate buffer (1 M 0.3 ml). Since the study of this enzymatic reaction took place in the presence of dextrins which did not contain phosphorus compounds, fractionation of saccharides of different sizes was carried out with alcohol without the use of barium. For this purpose, to a 1.5 ml sample taken before or after 2 hours and 30 min of incubation we added one volume of 10% trichloroacetic acid (TCA), removed the precipitated proteins, and to the TCA extract (2 ml) added 4 volumes of ethyl alcohol, mixed carefully and allowed to stand overnight, after which the precipitate which separated was centrifuged, washed with alcohol of the same concentration (10 ml) and after removal of the alcohol was dissolved in 0.1 N HCl (3 ml). In this solution dextrins were determined by their reducing power before and after acid hydrolysis, color of the sample with iodine, and investigation of the dextrins by paper chromatography and electrophoresis. The main alcoholic extract and the washings from the precipitate were combined, evaporated on the boiling water bath, and the residue was dissolved in 0.1 N HCl (15 ml) and the solution, which contained the lower molecular-weight dextrins, was fermented by special yeasts to remove the glucose (S. pombe Linder) and studied essentially by the same methods as in the analysis of the precipitate.

Those preparations are considered more active which form the greatest amount of polymerized glucose in the extract and in the precipitate.

The preparation of α-dextrins which was used in this reaction as a substrate was prepared by the action of salivary amylase on a purified glycogen preparation. For this purpose a mixture containing 3 g of glycogen, 40 ml of a saliva solution (1:5) and 100 mg of NaCl was incubated for varying periods of time. After inactivation of the amylase by acidification, the free sugars were removed from the incubation mixture by a special yeast; we used the above-mentioned strain for removal of glucose and S. ellipsoideus for fermentation of the maltose. After 4–6 hours the yeasts were centrifuged out and the protein which precipitated from the solution when TCA was added to the sample was discarded.

In Table 1 we give some data of the analysis of four preparations of α-dextrins, of which three (experiments 2, 3, 4) were obtained by the process described above. In the left side of the Table, we give the results of reduction by a definite weight of preparation before and after acid hydrolysis, on the basis of which we can estimate the aver-

age chain length of the saccharides in the preparation. In the right side of the Table we give data on their amount in the extract when 4 volumes of alcohol are added to the solution. We see that preparation No. 1 contains oligosac-

TABLE 1

Study of α-Dextrin Preparations

Expt. No.	Reduction in mg glucose								
				Alcohol extract			Precipitate		
	Before acid hydrolysis (a)	After acid hydrolysis (b)	Ratio a/b	Before acid hydrolysis (a)	After acid hydrolysis (b)	a/b	Before acid hydrolysis (a)	After acid hydrolysis (b)	a/b
1	2,041	5,000	1:2,45	2,100	5,081	1:2,47	0	0	0
2	2,856	7,860	1:2,7	2,790	7,500	1:2,5	0,060	0,300	1:5,0
3	2,628	9,120	1:3,4	2,662	6,620	1:2,48	0,252	2,424	1:9,6
4	13,800	3,012	1:4,5	2,560	7,360	1:2,4	0,564	5,204	1:9,2

charides whose average chain length is 2.47 and, with fractionation by alcohol, these oligosaccharides pass fully into the alcohol extract. The dextrins of preparation No. 2, obtained by the action of salivary amylase on glycogen during four days (before stopping the increase in reduction) remain almost fully in the alcohol extract after fractionation, and have about the same chain length as the previous preparation. Only a small part of the dextrins of this preparation (about 1/9) has a greater length (equal to 5) and is precipitated by alcohol. Preparation No. 3 which was obtained by the action of amylase for a shorter period of time (about a day) is a mixture of dextrins, part of which is precipitated by alcohol, and which has a considerably greater average chain length than the preceding (9.6). However, in this case most of the saccharides of this preparation which enter the alcoholic extract have a size similar to the previous preparation which shows about the same molecular weight (average). Preparation No. 4, which was submitted to the action of amylase for three hours only, contains considerably more high molecular-weight dextrins which are precipitated by alcohol. However, more than half of the dextrins of this preparation pass into the alcohol extract, with the same approximate chain length as in the previous case.

In the study of the transglucosylase activity of the liver we used all these preparations as substrates. The study showed that in the presence of these preparations there occurred formation of large molecule and small molecule oligosaccharides. However, the synthesis of high molecular-weight saccharides was most obvious in preparations No. 1 and 2, since here reduction after acid hydrolysis of the starting samples of precipitate was very slight.

TABLE 2

Study of the Activity of Enzyme Fractions

Incubation mixture: 3 ml of enzyme preparation + 1.5 ml of α-dextrins + 40 mg of glucose + 0.2 ml of acetate buffer. Increase in reduction given in mg of glucose per 1 ml of solution of the precipitate after acid hydrolysis

Experiment No.	Weight of crystals			Soluble enzyme No. 1			Soluble enzyme No. 2		
	Time of incubation, min								
	0	30	120	0	30	120	0	30	120
1	0	0.058	0.210	0	0.100	0.310	0	0.020	0.100
2	0	0.070	0.120	0	0.090	0.200	0	0.050	0.150
3	0	0.162	0.370	0	0.180	0.400	0	0.070	0.200

Method of Isolating Transglucosylase from Liver. The following is the method which we have worked out. Immediately after the rabbit was killed and exsanguinated, the liver was removed and homogenized for 2 min with 3 volumes of salt solution suitable for transformation of glucose into glycogen [3], a liter of which contains 4.01 g $MgCl_2$, 2.2% $CaCl_{29}$, 5.2 g KCl, and 4.01 g $KHCO_3$. The tissue was separated by centrifuging and discarded; the extract was passed through a layer of paper pulp on a Buchner funnel connected with a water pump

and then the extract was treated with a saturated solution of $(NH_4)_2SO_4$ made alkaline to pH 8.5 (to 0.6 saturation). The mixture was well stirred with a glass rod in a glass vessel for several minutes and allowed to stand in the cold overnight. On the next day the crystalline precipitate was separated by centrifuging at a low rate of speed (it settled well). The precipitate was washed 2–3 times with cold distilled water made alkaline with NaOH to pH 8.5. The washed precipitate consisted of crystals of well-defined rods with blunt edges, separate or clumped together, and with a bluish color. The crystalline precipitate had specific activity either as a suspension or in dissolved form.

TABLE 3

Some Analytical Data on the Crystalline Preparation of Transglucosylase

Experiment No.	Yield		Composition			Component salts			
	Weight of liver sample, mg	Weight of crystalline preparation, mg	Ash	Protein	Carbohydrate	Magnesium	Calcium	Ammonia	Phosphorus
			In percent						
1	100	195	76	4.1	0.6	18	0.95	12.8	22
2	98	196	76.4	4.2	0.59	19	0.7	13.4	23.8
3	108	200	77	4.0	0.58	19	0.9	13.4	23.8

Solution of the crystals could be produced in two ways:

1. In acetate buffer at pH 4.8, – in which a portion of the crystals, isolated from about 40–50 g of liver, was placed in 3 ml of acetate buffer, pH 4.8–5.0 and mixed. When the reaction medium had been established (which sometimes required the addition of several drops of 5 N HCl to the buffer), the crystals lost their form and dissolved, leaving a small residue of "skeleton", an amorphous residue which was centrifuged off and discarded (the preparation so obtained (Table 2) was called "soluble enzyme No. 1").

2. The crystals were dissolved in 0.6 N $(NH_4)_2SO_4$ at pH 4.8, when the protein enzyme precipitated. The precipitate was centrifuged off and extracted with acetate buffer at pH 4.8–5.0 for 40–60 min in the cold with stirring, then the insoluble part was removed and the enzyme solution appeared as a colorless, slightly opalescent liquid (the preparation thus obtained is called in Table 2 "soluble enzyme No. 2").

In the first process the dissolved enzyme preparation from the crystalline precipitate contained part of the salts which accompanied it and which dissolved, but the greater part of them remained as an insoluble residue. When the crystals were dissolved with a solution of $(NH_4)_2SO_4$ the enzyme preparation contained almost none of these salts. Table 2 gives the results on the activity of three preparations, studied as a suspension of the crystals and their solutions.

The results show that all three enzyme preparations are active since, when they are incubated with α-dextrins and glucose, we find an increase in glucose residues in the precipitate and extract (after acid hydrolysis). However, when we compare enzyme preparations with each other, we see that the soluble enzyme preparation obtained by solution of the crystals in $(NH_4)_2SO_4$ is the least active, which may be because in this process the treatment removes those salts which are activators for the enzyme.

Study of the Crystalline Enzyme Preparation. The study of the crystalline precipitate was carried out on preparations dried in a vacuum desiccator over P_2O_5. In separate samples we determined the moisture of the preparation dried in the desiccator, and all the determinations were calculated on the weight of dry residue. For removal of the protein, a sample of the preparation was dissolved in TCA. The Mg was determined according to Gadient, Ca according to Clark, NH_4 by the acidimetric method, P according to Fiske-SubbaRow, ash by the weight method,* nitrogen by Kjeldahl, carbohydrates by reduction in the solution before and after acid hydrolysis. The result of these investigations is given in Table 3 which shows chiefly that the yield of crystalline preparation from 100 g of liver in all three experiments is relatively constant at about 195–200 mg.

* The determination was carried out in the Institute of Inorganic Chemistry, Academy of Sciences, USSR.

The investigation of the composition of the crystals showed that they contain up to 76–77% salts, 4% protein, and a slight amount of carbohydrate (0.6%).

The initial experiments on the salt composition of the crystals showed that crystal formation occurred only in the presence of definite salt ions. The experiments were carried out in this way: equal weights of liver tissues were extracted with a salt mixture which contained the four salts, and with similar solutions from which one of the salts had been excluded. The study showed that the crystals are formed only if the ions Mg^{++} and NH_4^+ are present; without the addition of these cations from the outside the formation of crystals does not occur. Experiments with the exclusion of Ca were indefinite, since in half the experiments the crystalline precipitate had markedly less specific activity in the absence of Ca than when it was introduced into the mixture.

From the results of these investigations we made a quantitative determination of the above ions in the crystals; as the data of the Table show, the solution from the crystalline precipitate contains Mg, Ca, NH_4, and P, and the amount of Ca is considerably less than of Mg.

Table 4

Study of the Activity of Transglucosylase and the Presence in its Preparations of Other Contaminating Enzymes. Incubation mixture: A) 3 ml enzyme + glycogen or glucose-1-phosphate; B) 3 ml enzyme + glucose-1-phosphate; C) 3 ml enzyme + 0.003 M glucose + 0.005 M ATP + 0.5 M NaF; D) 3 ml enzyme + 1.5 ml α-dextrins + 40 mg glucose + 0.2 ml M acetate buffer

Preparation no.	A, phosphorylase						B, phosphoglucomutase		C, hexokinase	D, Transglucosylase			Protein in mg in 1 ml of solution of preparation
	Phosphorylytic activity		Phosphorylase action	Synthesizing action		Action of phosphorylase	Transformation of G-1-P into difficultly hydrolyzable P,%		Labile ATP P transferred to glucose, μg	Increase in reduction in mg glucose in alcohol extract after acid hydrolysis			
	Before incubation	After 1.5 hrs. incubation		Before incubation	After 1.5 hrs. incubation		Before incubation	After 1.5 hrs. incubation					
	P in μg			P in μg									
1	100	100	None	71	72	None	0	0	0	0	0.200	0.400	0.162
2	150	148	"	60	62	"	0	0	0	0	0.125	0.300	0.812
3	268	270	"	80	79	"	0	0	0	0	0.050	0.170	0.510

A study of the purity of the washings from the crystalline precipitate shows that they do not contain phosphorylases, phosphoglucomutases, nor hexokinases, enzymes whose presence in the preparations would be an obstacle for the study of the enzymatic reaction which interests us (Table 4). The study was carried out with preparations dissolved in acetate buffer and which had a definite activity, as the data of the Table show. In the last column of the Table we give the protein content in the preparations which we studied. Determination of hexokinase activity was carried out by the method which we have described [4].

DISCUSSION OF RESULTS

The investigation showed that our method of obtaining transglucosylase of the liver as a crystalline preparation is simple and rapid to use and is characterized by good reproducibility. The enzyme preparation is chiefly a crystal mass of magnesium ammonium phosphate which contains the protein enzyme. The yield of protein is of sufficient size with small variation: from 100 g of liver we isolated 200 mg of crystalline precipitate (as a dry residue) which contains 4% protein.

Study of the elements found in the composition of the crystals shows that Mg, NH_4, and P are present in nearly the same ratio as in the salt $MgNH_4PO_4 \cdot H_2O$. Probably Ca forms an analogous salt ($CaNH_4PO_4$) which is mixed in small amounts with the $MgNH_4PO_4$.

The content in the crystals of specific enzyme protein shows that the process of deposition of the protein evidently occurs as a result of its specific adsorption on this salt. The fact that washing the precipitate 3–5 times with water(made alkaline)does not cause loss in specific activity is one of the reasons which supports the correctness of our idea.

The solubility of the crystals in an acid medium allows us to obtain the enzyme as a solution, and when the crystals are dissolved in a solution of $(NH_4)_2SO_4$, the enzyme preparation does not contain salt mixtures, which are present in small amounts in the first preparation.

The method described in the present communication for isolating the enzyme adsorbed on a crystalline salt is analogous to the process published earlier for obtaining phosphorylase from muscle [5]. In the latter case the enzyme was also contained in a crystalline preparation which resembled $MgNH_4PO_4$ in a number of properties, and it was isolated in a very pure state. The purity of the preparation, established for both enzymes, characterizes this process as very promising, since it permits obtaining preparations of enzymes without accompanying mixtures of other enzymes which are usually an obstacle for studying the given enzymatic reaction.

SUMMARY

We have described a method for determining the activity of transglucosylase of liver. We have worked out a method for isolating this enzyme from liver, as a crystalline preparation, which permits getting the enzyme in a highly purified state.

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AN ULTRAVIOLET DENSITOMETER

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When various substances are separated by paper chromatography or paper electrophoresis, in many cases, especially in biology and medicine, we deal with materials which have an ultraviolet absorption spectrum.

Usually, in the quantitative determination of the substances which have been separated, we resort to coloring them by some reagent to indicate their position on the paper. Later quantitative determination of these substances is carried out by one of the known methods, using elution, photographic or densitometric methods.

Each of these methods has its advantages and disadvantages, but the densitometric method is the most generally preferred. All the same, the necessity for preliminary coloring of the separated substances even in this case complicates the method for quantitative determination and requires the expenditure of much time. Also, in a number of cases, the methods of coloring are unsuitable, because of absence of specificity for a given substance or group of substances by the reagent, and also in the case where the separation is carried out preparatively and coloring of the isolated substance is undesirable.

Therefore it is especially important to solve the problem of quantitative estimation of substances by the size of their respective absorptions directly in the ultraviolet spectrum. The use of a spectrophotometer for this purpose is limited by the comparatively high cost of this instrument and the need for special adapters for work with paper strips.

A step forward in the solution of this problem was the ultrachemiscope, worked out by Brumberg [1], an apparatus for chromatography and chemical analysis in ultraviolet rays.

The principle of the ultrachemiscope was based on the fact that substances which absorb ultraviolet, form shadows on a special fluorescing screen whose fluorescence is produced by ultraviolet illumination. Therefore, when the paper with the substances distributed on it is laid along the screen it is possible to determine exactly the position of these substances and permit a visual qualitative estimation.

The detection of uncolored substances in the ultraviolet in chromatographic work was also worked out by Tiselius and other authors by photographing a chromatographic column through a special liquid light filter. Tiselius carried out visual observations with a television apparatus with a special attachment, sensitive to ultraviolet light, whose construction was worked out by RCA (USA). However, this apparatus, also, in spite of its relative complexity, still did not solve the problem of express quantitative estimation of the separated substances.

On the basic principle used in the ultrachemiscope we have attempted to present an apparatus for objective and rapid quantitative estimation of substances separated by the methods mentioned, which can absorb in the ultraviolet.

In the present work we give a short description of the construction of an apparatus designed by us for this purpose, which we have given the abbreviated designation Type UDSh-1, and we give some examples which illustrate the possibilities for its practical use.

In the basic work on the UDSh-1 densitometer, the position of the absorption of the different substances in the ultraviolet was measured with a bactericidal lamp of type BUV-30-P. The measurement was made only after transformation of the energy of the ultraviolet rays into visible illumination by the use of a special lumi-

nescent screen. As the indicator of the beam of light which illuminated the screen, we used a photoelement with an external photoeffect, with an antimony-cesium cathode.

Since the measured beam of light is especially small and the generated photocurrent cannot be measured directly by an ordinary galvanometer, we amplified the photocurrent with an electron tube. For this purpose we used an amplifier, somewhat modified by us, taken from a "Cactus" microroentgenometer.*

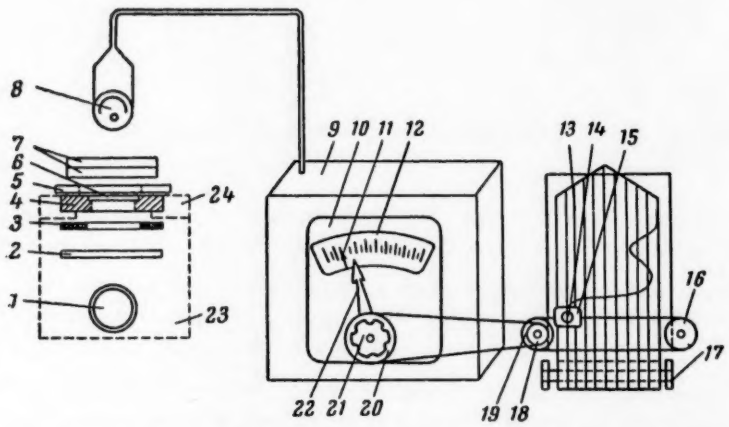

Fig. 1. Principle of the UDSh-1 densitometer; 1) Source of ultraviolet light (BUV-30-P lamp); 2) UFS-1 light filter; 3) diaphragm; 4) guiding rods; 5) fluorescent screen; 6) paper strip; 7) light filter; 8) photoelement; 9) amplifier; 10) milliammeter; 11) arrow pointer; 12) scale; 13) diagram paper; 14) pen; 15) sliding pen holder; 16, 18, 19, 20) pulleys; 17) spool for diagram paper; 21) knob for "observation" mechanism; 23) case for the BUV-30-P lamp; 24) cover for case.

Measurement of the light beam in the UDSh-1 densitometer was carried out by direct recording of the photocurrent on a milliammeter built into the front panel of the amplifier.

The principle of operation of the UDSh-1 densitometer is given in Fig. 1.

The bactericidal lamp 1 is placed in a special case 23. In the cover 24 of the case a groove is made, into which are inserted the changeable guiding rods 4 with widths of the slot of 10, 20, and 30 mm. The rods serve to fix the position of the paper strip 6 as it moves along during the measurement.

For the passage of the ultraviolet illumination, an opening is made in the cover of the case and in the guiding rods and is covered with the diaphragm 3. The isolation of the ultraviolet light and the absorption of the visible light is carried out with the light filter 2, type UFS-1.

The paper strip is placed in the grooves of the guiding rods and held to them by a carriage in which is placed the fluorescent screen 5. In the central part of the carriage is an opening through which the measurable light from the screen passes to the photoelement 8, which is connected to the amplifier 9 by a flexible, insulated cable.

For recording the values we can use a recording galvanometer or an automatic potentiometer of type EPP-09.*, attached to a special terminal of the amplifier. However, the high cost of this apparatus induced us to the simpler principle of a semiautomatic recorder, suggested to us by the earlier construction of a densitometer of type PDSh which works in the visible field [2, 3]. In this case the recording of the measured value was carried out by a manual method with the help of the so-called "observation mechanism". This consists of a system of pulleys which on one side are connected by a steel wire or cord to the "observation" pointer 22, and on the other to the recording pen 14.

* Manufactured by the Ministry of the Radiological Industry, USSR.

* Electronic potentiometer EPP-09. Instructions for Mounting and Use. Oborongiz, 1951. [In Russian].

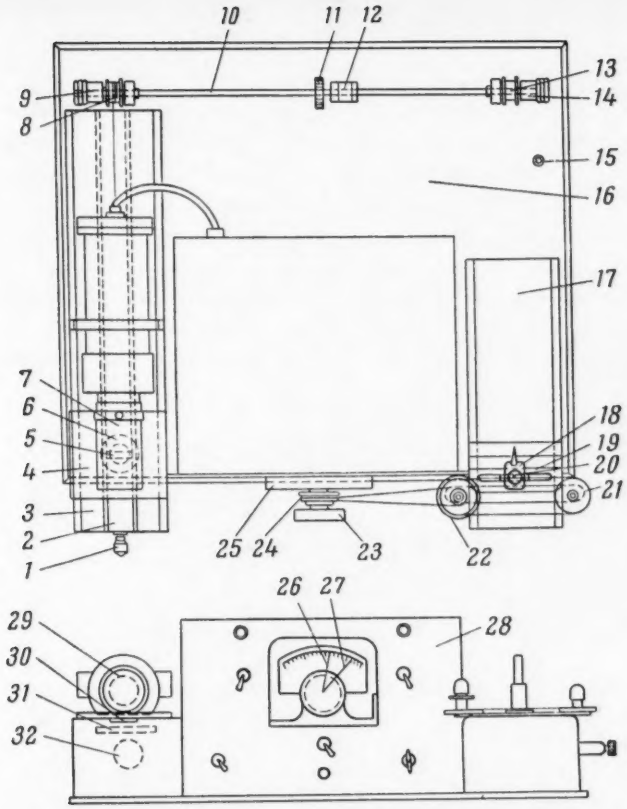

Fig. 2. General form of the UDSh-1 densitometer. 1) diaphragm handle; 2) guiding rods; 3) case for the BUV-30-P lamp; 4) holder for fluorescent screen; 5) slit; 6) fluorescent screen; 7) photoelement chamber; 8, 13) drive spool; 9, 14) handles for adjusting the connections; 10) gear axle; 11) reducing gear; 12) electric motor; 15) switch for motor; 16) mounting base; 17) case for diagram paper; 18) sliding carriage for recording apparatus; 19) guides for carriage; 20) pen; 21, 22, 24) pulleys; 23) handle for observation mechanism; 25) milliammeter; 26) indicator arrow for milliammeter; 27) pointer for observation mechanism; 28) amplifier; 29) photoelement; 30) paper strip; 31) light filter UFS-1; 32) BUV-30-P lamp.

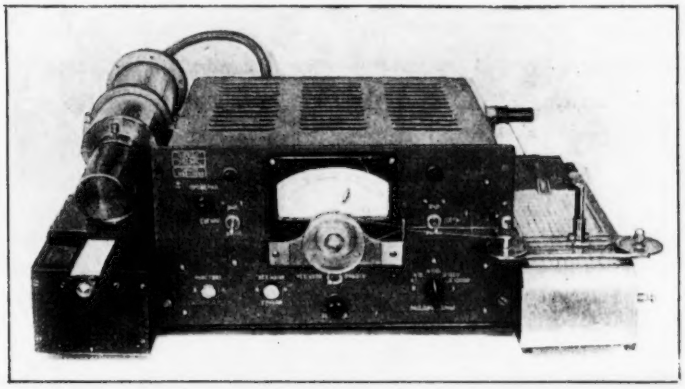

Fig. 3. External form of UDSh-1 densitometer.

When the observation pointer is moved (by hand) with the shifting pointer 11 of the milliammeter 9, it produces a corresponding shift in the recording pen 14 of the recording mechanism and this gives the graph on the diagram paper.

The synchronizing of the shift of the diagram paper and the paper strip with the separated substances on it is carried out with a small electric motor and a corresponding reducing gear. There are two drums on the axle of this gear which are connected by cord or wire to the diagram paper and the paper strip, and which control their movements.

The first passage of the light of the fluorescent screen (100% passage of the light through the paper) to the cathode of the photoelement is produced by the slit diaphragm 3, when the light is passed through the "clean" (without substance) part of the paper strip as it passes over the cover of the case which contains the ultraviolet source. When the paper strip passes along the slit diaphragm, the substance on the strip absorbs the ultraviolet light which strikes the fluorescent screen and thus lessens the intensity of fluorescence of the screen. The photoelement 8 reacts to this change in the beam of light which strikes its cathode and this is observed on the milliammeter 10.

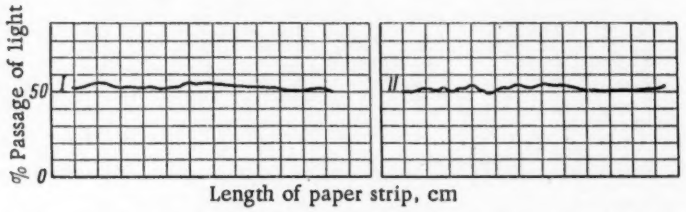

Fig. 4. Effect of "cloudiness" of chromatographic paper on the results of the measurement: I) Schleicher and Shull paper No. 2045; II) Leningrad paper No.2.

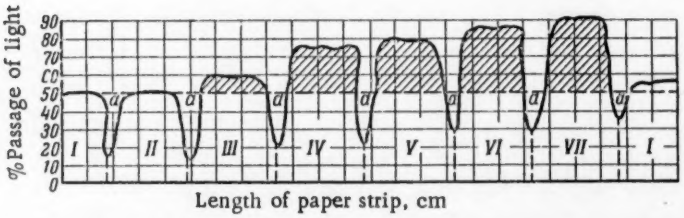

Fig. 5. Effect of different "washing" solutions on the absorption of ultraviolet light by Leningrad paper No. 2. I) without washing; II) washing with 0.5% trylon (versene); III) 12% acetic acid; IV) 0.2% solution of 8-hydroxyquinoline in 70% alcohol; V) 1% alcoholic HCl solution + 0.1 N NaOH; VI) 20% formic acid; VII) Hydrofluoric acid + HCl ; a) absorption of illumination at points where different pieces of paper are glued together.

During the measurement, rotation of the knob 21 in the "observation mechanism" keeps its pointer 22 continuously in the same position as the indicator arrow 11 of the milliammeter. This automatically causes the pen 14 to trace the graph of the absorption of the substance being measured. Then quantitative calculations can be carried out by planimetric measurements and determination of the area traced on the abscissa and by the resulting graph of absorption of the different substances. The especially small difference in the intensity of the light during the measurement requires the use of considerable amplification in measurement of the photocurrent and demands stabilization of all the measuring devices of the apparatus.

Fig. 2 shows the arrangement of the various parts of the apparatus, and Fig. 3 shows its external appearance.

The UDSh-1 densitometer was given a thorough testing. First we used it to study the absorption characteristics of different types of chromatographic paper.

We showed that the magnitude of the errors caused by nonhomogeneous stucture ("cloudiness") in the paper itself in the most unfavorable case which we studied did not exceed±4%, as Fig. 4 shows. In most cases, the magnitude of this error was actually less.

TABLE

Comparison of the Quantitative Determination of Purine Nucleotides by the Method of Elution and the Densitometer

Substance	Amount of substance determined by elution method, %	Amount of substance determined on densitometer, %	% Difference	Substance	Amount of substance determined by elution method,%	Amount of substance determined on densitometer, %	% Difference
Chromatogram 1 (Fig. 8)				Chromatogram 2 (Fig. 9)			
GPP*	8.2	7.4	−2.4	GPP*	8.9	9.1	+ 0.6
ATP	26.2	26.0	− 0.6	ATP	26.4	27.6	+ 2.4
ADP	32.8	33.3	+ 1.5	ADP	33.4	31.8	−4.8
AMP	32.8	33.3	+ 1.5	AMP	31.3	31.8	+ 2.1

* Mixture of guanosine polyphosphates (guanosine di- and triphosphates).

At the present time it is well known that the presence of different substances in the paper itself, the so-called "contaminants" is sharply changed in the process of electophoretic or chromatographic treatment of the paper, since these substances have the ability to shift along the paper like the substances which are being separated in the mixture under study. Therefore strictly quantitative determinations require corresponding preliminary treatment of the paper by different solvents.

A study on the densitometer of the degree of "purity" of the paper attained as a result of preliminary washing, carried out according to Lyubimova, showed that a suitable choice permitted rapid obtaining of an objective quantitative estimation on chromatographic paper which was first washed. The results of this series of such measurements are given in Fig. 5.

In Figs. 6 and 7 we give examples of quantitative measurement of inosinic acid, placed on the paper in amounts of 50 and 100 μg. The density obtained on the densitometer diagram gave a ratio of 1:2, which is proportional to the concentration of substances placed on the paper.

Figs. 8 and 9 illustrate the separation of mixtures of purine nucleotides by paper chromatography. In these figures we give a photograph of the chromatogram taken in ultraviolet light and a graph of the absorption of the separated substances obtained on the UDSh-1 densitometer. In the Table we have shown the results of quantitative measurement of these substances by the elution method and the results obtained with the densitometer; the maximum difference in the determinations is 4.8%.

At the present time we have carried out further studies on this densitometer, but the results obtained already show that this instrument can be used for the study of substances separated by paper electrophoresis or chromatography.

For valuable consultations and participation in experiments on the densitometric determination of the effectiveness of washing out chromatographic paper, we are grateful to M. N. Lyubimova; we also thank A. A. Baev and T. V. Venkstern for the chromatograms of nucleotides and the quantitative data on spectrophotometric determination of the different nucleotides isolated by the elution method.

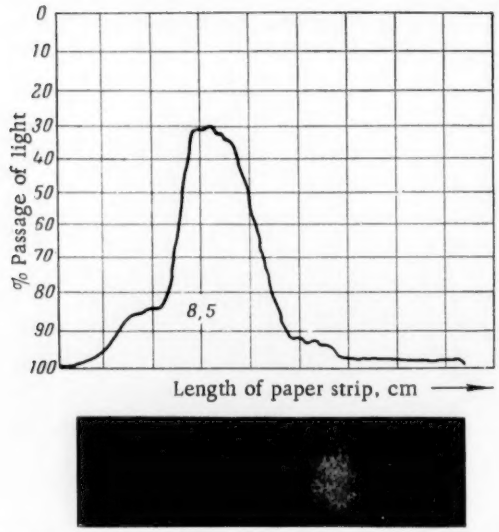

Fig. 6. Graph of absorption of inosinic acid (50 μg), obtained on the densitometer. 8.5 – area in planimeter units.

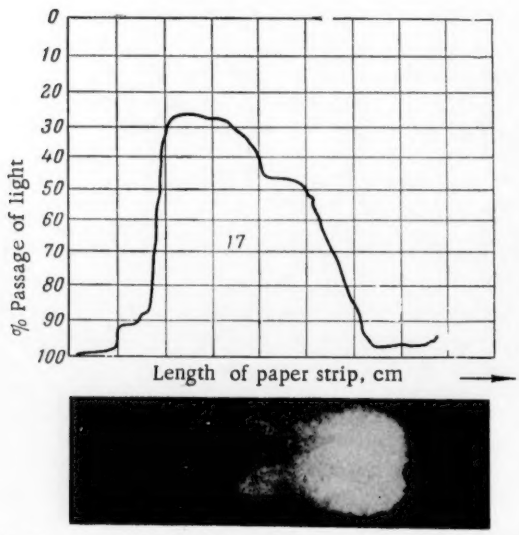

Fig. 7. Graph of absorption of inosinic acid (100 μg). 17 – area in planimeter units.

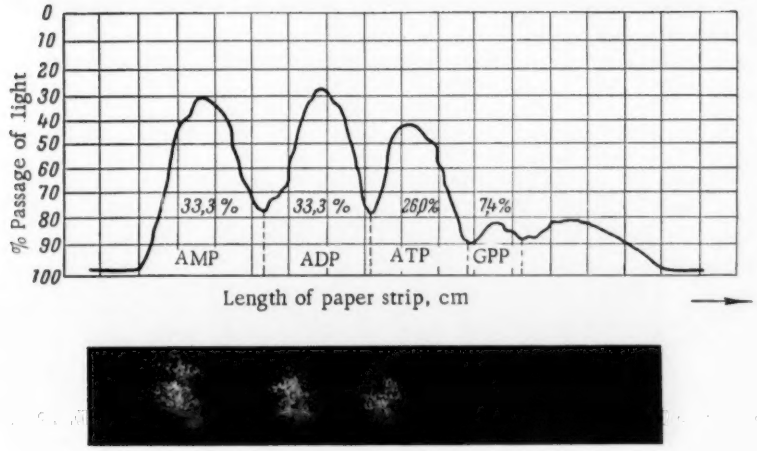

Fig. 8

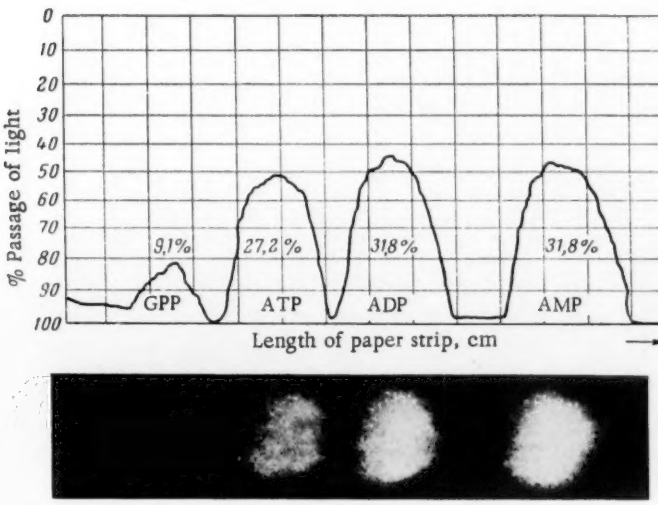

Fig. 9

Figs. 8 and 9. Photographs of chromatograms of purine nucleotides in ultraviolet light and graphs of their absorption obtained on the UDSh-1 densitometer.

SUMMARY

A new construction of the registering densitometer of the UDSH-1 type has been developed for a quantitative assay of compounds absorbing within the UV-range of the spectrum and partitioned on paper by electrophoresis or chromatography. Measurements by means of the above densitometer do not require any preliminary treatment (staining) of the paper.

Examples are presented illustrating the use of the densitometer in question and indicating the range of its practical application.

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* Original Russian pagination. See C. B. Translation.

NITROGENOUS SUBSTANCES OF THE MUSCLE OF ONE-YEAR-OLD CARP DURING HIBERNATION

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We have shown earlier that during hibernation of one-year-old carp under natural conditions there is a decrease in amount of protein in blood serum, and in total nitrogen, the water content increases and the content of organic substances, protein and lipid, in muscle is decreased [1]. The present paper reports a study of the nitrogenous substances of extracts and the amino acid composition of proteins of muscle in year-old carp at the time of winter starvation under natural conditions and in the aquarium.

The literature on the chemistry and metabolism of nitrogenous extractives in fish is limited to the work of Sh'yun [2] and Yudaev [3, 4]. Incomplete information on the amino acid composition of the muscle proteins of carp was obtained in 1919 by Okudo (cited by Reikhart [5]). The essential amino acids in muscles of one-year-old carp were studied by Karasikova and Petrenko [6].

METHODS

For the determination we used muscles of one-year-old mirror carp grown in the ponds of the "Red Banner" State Farm in the Moscow region. In the autumn we collected fish weighing 20–35 g and transferred them to a hibernation tank, from which some of the fish were moved in December to an aquarium under conditions of starvation. For each study at definite intervals we took for analysis 15–20 fish from the hibernation tank and the aquarium. Total nitrogen was determined by the Kjeldahl method. Protein from the muscle homogenate was precipitated by 5% TCA, centrifuged off, and defatted, dried to constant weight, and the protein nitrogen determined in it. Using the methods described in the literature mentioned below, we studied in the supernatant liquid the nitrogenous substances which were soluble in TCA: amino nitrogen, ammonia, amino acids, creatine and creatinine [7], and components of the adenylic system [8]. The proteins were hydrolyzed with 20% HCl. The amino acids of the extract and hydrolyzate were determined by the chromatographic method of Bode [9]. *

RESULTS

Chemical Analysis of the Nitrogenous Substances. In Table 1 we give the results of the analysis of the nitrogenous substances of muscles in the fish during starvation under natural conditions and in the aquarium. As Table 1 shows, during the winter (December – March) there is a decrease in content of total and protein nitrogen of the muscle. At the same time there is a decrease in amount of amino nitrogen chiefly because of decreased content of amino acids and creatine, and also the nitrogen of the components of the adenylic system. The amount of ATP in December was almost twice as low as before, and at the end of February it began to increase, reaching the original values in March. At the end of April and the beginning of May the amount of ATP fell again.

In the spring period of starvation, from the end of April or the beginning of May, the state of the nitrogen balance in the fish seemed rather unusual. The amount of total nitrogen in the muscle rose somewhat. The nitrogen of the muscle protein in April and the beginning of May did not decrease. This increase in amino nitrogen occurred chiefly because of creatine. The amount of creatine nitrogen at the beginning of May reached the original value, 170.7 mg.

*N. Kazarovets, E. Kolenchuk, M. Syrova and A. Chukhrova assisted in carrying out the experiments.

In the spring, when the water temperature rose, the fish prepared for the growing period of life, and, naturally, the number of muscular movements increased. It is safe to assume that at this time the composition of the blood will show an excess of nitrogenous substances because of the products of breakdown of proteins of the internal organs, so that the breakdown of proteins in the muscles will be somewhat limited.

TABLE 1

Nitrogenous Substances of Muscle of One-Year-Old Mirror Carp During Hibernation per 100 g Dry Weight

Date of analysis	Total nitrogen, g	Protein nitrogen, g	Amt. of g protein g (N · 6.4 *)	Nitrogen of extractives, mg								
				Amino	Amino acid	Creatinine	Creatine	Ammonia	ATP	ADP	AMP	Unknown compound
3.X 55	2,94	2,59	16,58	348	155,5	170,5	3,8	6,9	4,2	1,8	1,0	5,2
24.XII 55	2,72	2,44	15,62	270	142,4	105,5	12,2	2,0	2,6	1,3	1,0	2,9
28. II 56	2,46	2,21	14,03	268	93,4	144,5	8,7	6,0	3,3	1,7	0,8	9,5
7. III 56	2,48	2,20	14,08	281	112,2	146,7	4,1	7,0	4,3	1,5	0,7	4,5
24. IV 56	2,55	2,25	14,40	301	109,2	168,3	3,8	7,5	2,4	1,2	0,6	8,0
8. V 56	2,53	2,24	14,33	290	102,2	170,7	6,5	2,2	2,6	1,2	0,8	3,7
15. III 56	1,69	1,46	9,34	235	103,3	109,0	7,4	2,4	2,2	1,3	0,9	8,5
20. IX 56	2,23	1,99	12,7	244	107,0	109,5	6,5	8,3	3,1	1,6	0,8	6,5

* The amount of nitrogen per 100 g of dry muscle protein in the carp, taken at different periods during hibernation remains constant and equals, on the average, 15.6%.

In long starvation there is certainly breakdown of proteins of the internal organs, but the amount of this, compared to muscle proteins, is slight.

In starvation in the aquarium (March 15, 1956) the amount of nitrogenous substances of muscle decreased to almost half (compared to the autumn): protein nitrogen by 50%, ammonia by 50%, creatine by 40%, amino acid nitrogen by 35%.

In Table 1 we give the result of analysis of the fish muscle on September 20, 1956. This group of fish was starved under natural conditions from October to December 24, and was then transferred to the aquarium and then brought to the greatest emaciation. At the end of March the fish were fed, at first with dry daphnia, and then with live midge larvae. After about a month the majority of the carp grew strong, became active, and took food well. However, their total growth was small. After five months of feeding in the aquarium the amount of protein in the muscles was 13% instead of 16.6% which had been observed the year before at the beginning of hibernation. The amount of amino acid and creatine nitrogen was also maintained at a relatively low level, that is, about 100 mg (instead of 150–170 mg). Thus, prolonged starvation of the fish led to irreversible damage to their metabolism, and limited growth and development of the whole organism.

Dynamics of Amino Acids in Carp-Muscle Extractives during Hibernation. The nitrogenous extractives in the carp, as in other fish, make up a rather small part of the total nitrogen of the muscle. The amount of nitrogen varies depending on time of the year and nature of the surroundings. According to our data, in October, before hibernation, the quantity of nitrogen of the extractives is 11.8% of the total nitrogen, in December 9.9% in February 10.8%, in March 10.1%, in April 11.8%, in May 11.4%, and after severe starvation in the aquarium, 13.8%. In well-fed fish, the free amino acids make up 5.8% of the muscle, and in fish after winter starvation, 4%.

The total amount of amino acid nitrogen in the muscle extracts (Table 1 and 2) for one-year-old carp at the time of winter starvation is decreased. Thus, at the beginning of winter (October 3) its amount is 155.5 mg per 100 g of wet tissue, at the end of December it is 142.4 mg, at the end of February 93.4 mg, at the beginning of March 112.2 mg, at the end of April 109.2 mg, and at the beginning of May 192.2 mg; that is, in the spring, the total level of amino acid nitrogen has fallen by $^1/_3$.

TABLE 2

Amino Acid Dynamics in Muscle Extracts of One-Year-Old Mirror Carp During Hibernation per 100 g of Dry Weight

Amino acid	3.X 55		24.XII 55		28.II 59		7.III 56		24.IV 56		8.V 56		15.III 56	
	N, mg	wt., mg	N, mg	wt., mg	N, mg	wt., mg	N, mg	wt., mg	N, mg	wt., mg	N, mg	wt., mg	N, mg	wt., mg
Cystine	4,4	37,7	6,3	53,5	3,7	31,4	4,7	40,0	4,4	37,7	2,2	18,9	1,4	12,0
Lysine	16,7	87,2	12,0	62,6	6,5	34,0	26,8	140,0	37,8	197,3	26,6	138,8	22,1	115,4
Histidine	36,6	135,0	27,7	102,2	14,1	52,0	11,9	43,9	9,1	33,7	13,9	51,3	7,8	28,8
Arginine	3,1	9,6	1,6	5,0	1,9	5,9	5,4	16,7	10,8	33,5	16,5	51,1	34,0	105,7
Aspartic acid	3,5	33,2	2,5	23,7	2,1	20,0	4,7	44,6	2,7	25,6	5,3	50,3	6,8	64,6
Glycine	45,7	245,0	41,6	223,0	34,0	182,2	31,6	169,4	26,3	141,0	26,7	143,1	13,4	71,8
Glutamic acid	11,0	115,5	8,5	89,2	2,8	29,4	5,7	59,8	3.6	37,8	2,3	24,2	6,7	70,3
Sarcosine	11,5	73,1	10,5	66,8	9,6	61,0	8,7	55,3	1,9	12,0	Следы		3,7	23,5
α-Alanine	23,0	146,3	31,7	201,6	18,7	118,9	12,7	80,7	12,6	80,1	8,7	55,3	7,4	47,4

In the Figure we give the distribution of the amino acids of the muscle extracts on paper as disclosed by ninhydrin. In Table 2 we give the results for their quantitative determination as nitrogen. The amino acids detected are cystine, lysine, histidine, arginine, aspartic acid, glycine, glutamic acid, α-alanine, and also sarcosine.

As the Figure and Table 2 show, the quantitative ratios of these amino acids change during winter starvation. The amount of cystine in October is 37.7 mg and in December, 53.3 mg; from February to April it varies from 30–40 mg, in May it falls to 18.7 mg, or a decrease of a half.

The behavior is unusual for lysine, histidine, and arginine, which follow each other very closely on the chromatogram.

At the beginning of winter, lysine was 87.2 mg; at the end of February its level dropped more than half, then its amount rose sharply and at the end of April reached a maximum, 197.2 mg. In May the amount of lysine fell somewhat, but its content remained high (138.8 mg), just as it did in starvation in the aquarium (115.4 mg).

The maximum amount of histidine at the beginning of October was 135.0 mg. During hibernation, its amount gradually fell. At the end of April the histidine level had fallen to 33.7 mg, that is, a more than fourfold decrease.

Arginine at the beginning of hibernation was found in the extract only in the amount of about 9.6 mg, in December to February, 5.0–5.9 mg, in March it rose to 16.7 mg, at the end of April and the beginning of May it increased 3–5 times (33.6 -51.1 mg). The increase in arginine in muscle extracts of fish starved in the aquarium was very striking (Table 2, March 15): its amount increased tenfold and reached 105.6 mg.

The amount of aspartic acid in October was 33.2 mg, in December and February it began to fall, and in March rose to 44.6 mg. In April, its level again fell to 25.6 mg, and at the beginning of May increased to 50.3 mg. In starvation in the aquarium the amount of aspartic acid continued to increase and reached 64.4 mg, that is, increased twofold.

Glutamic acid behaved differently. At the beginning of hibernation its amount was 115.5 mg; during the winter period its level fell and in May reached 24.4 mg, that is dropped almost fivefold.

More glycine was contained in carp-muscle extract than any other amino acid. At the beginning of October we found about 245 mg of it. During the winter the amount of glycine gradually fell and at the end of May was 143 mg, almost a twofold drop.

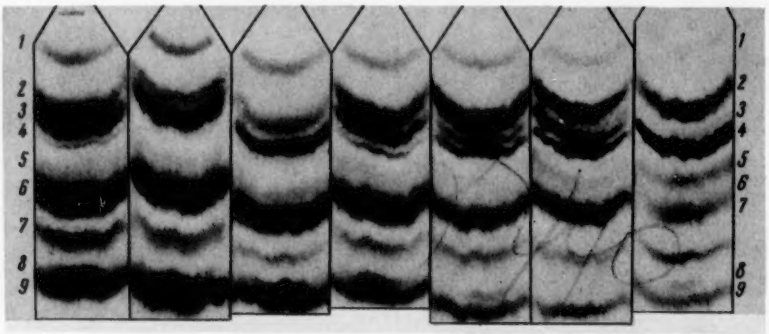
Table 3

Amino Acid Composition of the Muscle Protein of One-Year-Old Mirror Carp during Hibernation per 100 g Dry Weight

Amino Acid	3. X 55		24. XII 55		28. II 56		7. III 56		8. V 56	
	nitrogen, g	weight, g	nitrogen, g	weight g	nitrogen g	weight, g	nitrogen, g	weight g	nitrogen g	weight, g
Cystine	0.180	1.56					0.20	1.73	0.20	1.73
Lusine	2.70	14.0	3.45	16.79	3.41	16.64	2.66	13.8	2.57	13.40
Histidine	0.75	2.77	–	–	–	–	0.76	2.87	0.85	3.14
Arginine	2.43	7.56	2.37	7.37	2.34	7.26	2.43	7.56	2.40	7.46
Aspartic acid	1.27	12.12	1.11	10.54	1.26	11.96	1.21	11.70	1.23	11.90
Glycine	0.48	2.6	0.45	2.50	0.46	2.49	0.45	2.40	0.39	2.06
Glutamic acid + threonine	1.84	19.33	1.88	19.60	1.85	19.42	1.86	19.42	1.90	19.95
Alanine	1.88	11.95	1.85	11.78	1.81	11.50	1.89	11.96	1.95	12.38
Tyrosine	0.26	3.34	–	–	–	–	0.26	3.34	0.26	3.38
Methionine	0.15	1.61	0.147	1.56	0.17	1.80	0.17	1.80	0.20	2.03
Valine	0.83	7.0	0.80	6.73	0.79	6.60	0.83	7.18	0.83	6.87
Phenylalanine	0.42	5.05	0.41	4.89	0.44	5.20	0.40	4.70	0.41	4.88
Leucine + isoleucine	1.44	13.5	1.43	13.43	1.42	13.20	1.45	13.50	1.41	13.10
Serine	–	Found, but not determined								
Tryptophan	–	Found, but not determined								
Proline	–	Found, but not determined								

Alanine also fell in the carp during the winter. At the beginning of October the amount of α-alanine in the muscle extract was 146.3 mg, and in May, 55.5 mg, that is, almost 3 times less.

In the composition of the muscle extract of the pond carp we always found an amino acid which we had not studied sufficiently; it always accompanied α-alanine, moving along with it on the descending chromatogram. Our preliminary study led us to believe that this was sarcosine. Its amount at the beginning of winter was 73 mg, falling in the spring, so that in May sarcosine appeared only in traces.



Sample on 10/3 | Sample on 12/24 | Sample on 2/28 | Sample on 3/7 | Sample on 4/27 | Sample on 5/8 | Sample on 11/15

Amino acids in carp muscle extracts during hibernation. 1) Cystine; 2) lysine; 3) histidine; 4) arginine; 5) aspartic acid; 6) glycine; 7) glutamic acid; 8) sarcosine; 9) α-alanine.

Thus, during winter starvation of the one-year-old carp under natural and artificial conditions we find two contradictory effects in the muscle extracts: on the one hand, a fall in the amino acids cystine, histidine, glycine, glutamic acid, α-alanine, and sarcosine; and on the other, an increase in the amount of lysine, arginine, and aspartic acid. The occurrence of this effect still remains unexplained. The observed increase in lysine, arginine, and aspartic acid evidently results from the decomposition products of muscle proteins. It will be shown below that the amount of these amino acids is greatest in the composition of the muscle-protein hydrolyzate.

Amino Acid Composition Of the Muscle Protein of One-Year-Old Carp During Hibernation. The results of the investigation of amino acid composition of muscle protein in carp with samples taken from the hibernation tank at the above periods are shown in Table 3.

We found 18 amino acids in the hydrolyzate from muscle protein. The greatestamount of nitrogen was found in lysine (2.7 g), arginine (2.43 g), and also α-alanine (1.88 g), glutamic acid along with threonine (1.84 g), leucine and isoleucine (1.44 g), aspartic acid (1.27 g), with comparatively little in valine (0.83 g), histidine (0.75 g), glycine (0.48 g), phenylalanine (0.42 g), and very little in tyrosine (0.26 g) and methionine (0.15 g).

During winter starvation, in spite of the decreased protein nitrogen in the fish muscles, their amino acid composition remains constant and their quantitative ratio does not show any particular change.

Our results do not agree with the conclusions of Petrenko and Karasikova [6] who consider that in the muscle composition of one year old carp,leucine and phenylalanine are absent and who note the full disappearance of valine and methionine in the wintering one-year-old carp. Chromatographic analysis of a number of hydrolyzates of muscle protein of one year old carp which we have carried out indicate the presence of leucine and isoleucine (13.47%), phenylalanine (5.05%), methionine (1.61%), and valine (7.0%). Their amounts are almost unchanged during the winter. Our data on the analysis of amino acids of carp-muscle protein in most cases agree with the results of the analysis of Okudo [5].

TABLE 4

Amino Acid Composition of Muscle Protein of Crucian Carp per 100 g Dry Protein

Amino acid*	After 9 mo. starvation in aquar.	After 9 mo. starving and 3 mo. feeding
	Nitrogen in g	
Cystine	0,24	0,22
Lysine	2,35	2,35
Histidine	0,88	0,86
Arginine	2,57	2,48
Aspartic acid	2,35	2,14
Glycine	0,55	0,55
Glutamic acid + + threonine	1,51	1,50
Alanine	1,95	1,95
Tyrosine	0,22	0,22
Valine + methionine	0,90	0,90
Phenylalanine	0,42	0,41
Leucine + isoleucine	1,38	1 50

*Serine, proline, and tryptophan were observed on the chromatogram, but were not determined quantitatively.

In Table 4 we give data on the analysis of muscle protein of crucian carp. The study was of the muscle protein of crucian carp which lived in the aquarium and which were exposed after 9 months starvation to the utmost emaciation. We also studied the muscles of crucian carp which after 9 months starvation were fed for 3 months on midge larvae and daphnia.

As the material which we present shows, in starvation,the crucian carp, like the carp,show a constant total amount of amino acids and their ratio in the muscle protein does not change. Thus, our results fully confirm the viewpoint expressed earlier by Fomin and Demin [10] and by Zbarskii and Stepanova [11] that feeding has no notable effect on the amino acid composition of organs and tissues of the living organism.

When we compare the chromatograms of the muscle protein hydrolyzates of crucian carp and carp we find the same amount of amino acids. There is a difference in the quantitative relation of some amino acids (Table 3, 4), for example: cystine in the crucian carp is 0.24 g, in the carp 0.18 g; aspartic acid in the crucian carp is 2.35 g, in the carp, 1.27 g. The amounts of other amino acids in the muscle-protein hydrolyzates of crucian carp and carp are very similar.

SUMMARY

During winter starvation under natural conditions and in the aquarium we find in the muscles of one-year old-carp a decrease in the amount of protein and some free amino acids occurring in the composition of the nitrogenous extractives of the muscle.

* Author's name not verified.

The amino acid composition of carp and crucian carp muscles during hibernation and starvation in the aquarium does not change appreciably. Feeding has no particular effect on the qualitative or quantitative composition of muscle protein in fish. During starvation we find in the muscle extract of carp a decreased content of cystine, histidine, glutamic acid, glycine, α-alanine, and sarcosine, while the amount of lysine, arginine, and aspartic acid is increased.

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ATP-ASE ACTIVITY AND CONTRACTILE PROPERTIES OF MYOSIN

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The occurrence of reciprocal interaction between myosin and adenosine triphosphate (ATP) was discovered by Engel'gardt and Lyubimova [1, 2]. These authors not only showed the presence of ATP-ase activity in the contractile protein of muscle (myosin), but also observed the sharp increase in elasticity (extensibility) of myosin fibers prepared and concentrated in the usual way, which, however, retained enzymatic activity [3]. They also showed that at physiological concentrations of KCl (0.16–0.17 M) the actomyosin gel (fiber) in the presence of ATP and $MgCl_2$ underwent syneresis, that is, isodimensional contraction [4, 5]. Under other conditions and nonphysiological concentrations of salt [6], enzymatically active actomyosin fibers with the addition of ATP could undergo swelling, anisodimensional contraction, or extension under a load. Thus it can now be considered to be established that the reaction of actomyosin and myosin gel (fiber) when ATP is added can differ depending on the conditions of the experiment.

Although at physiological salt concentrations the characteristic reaction with ATP for actomyosin fibers is syneresis, it is still important to note that other reactions of actomyosin and myosin gel (fiber) when ATP is added, observed under changed experimental conditions, also require for their appearance the retention of ATP-ase activity by the contractile protein.

Evidently, the models suggested by Engel'gardt and Lyubimova [2], Torbochkina [6] and others, as well as the models described by Szent-Gyorgyi [4, 5] are quite suitable for demonstration of the connection between enzymatic activity of myosin and the change in its internal structure (physical state) during reaction with ATP. This question has been treated in more detail in another paper [7]. Buchthal and co-workers [8] have come to another conclusion; they have shown the possibility of contraction of actomyosin fibers in reaction with ATP, apparently without ATP-ase activity. For this the Danish authors used myosin obtained according to the description of Singer and Meister, [9] which in their opinion had practically no ATP-ase activity. Thus, it follows from the paper of Buchthal and co-workers that the ability of actomyosin gel to undergo syneresis in the presence of ATP is not connected directly with enzymatic (ATP-ase) activity of the protein. Evidently this conclusion, if it is true, will have great significance. In their work, however, there are facts which force us to believe that the preparation which Buchthal and co-workers [8] used was not completely lacking in ATP-ase activity. Actually, a myosin preparation which fully lacks ATP-ase activity but retains the inherent myosin hydrophilic nature and ability to dissolve in 0.6 M KCl, loses its ability to form a complex with actin; actomyosin also, after loss of ATP-ase activity, loses all its most characteristic contractile properties, whether it is in the form of the actomyosin fiber or remains in the condition of a muscle fiber.

Some years ago, one of us [10] showed that under the influence of high pressure (4000 atm) myosin, in distinction from potato apyrase and water-soluble ATP-ase isolated from the smooth muscle of the uterus, was inactivated, and, as we assumed, denatured. In the present work we give data which show that solutions of purified myosin under the influence of high pressure actually fully lose their ATP-ase activity, but the protein still remains almost entirely in solution.

METHODS

Obtaining purified myosin. Rabbits were killed by air embolism and then exsanguinated. The protein was removed from the muscles of the hind legs. The muscle was carefully freed from fat and connective tissues, cooled with ice, and passed twice through a previously cooled meat grinder. Three times the volume of Weber solution was poured over the mince. The resulting preparation was stirred energetically in the cold for 7 min and then centrifuged for 20 min at 3000 r.p.m. Ten volumes of cold water was added to the centrifugate and the pH of the solution was brought to 6.8 by adding 3% acetic acid. After 2–3 hours the precipitate which settled out was centrifuged and washed once with cold water. We carefully added to the precipitate a 0.02 M K_2CO_3 solution (containing 10 ml of 1% phenolphthalein per liter) with careful stirring until the solution had a stable rose color (pH 8.3). Then we added a 2 M KCl solution to a final concentration of 0.5 M. The resulting solution was diluted at room temperature with 10 times its volume of water, first made alkaline with K_2CO_3 to a weak rose color with phenolphthalein. The resulting pasty precipitate of actomyosin was separated by centrifuging anddiscarded. The opalescent, rose-colored centrifugate was cooled and treated with an equal volume of cold water and the pH was adjusted to pH 7.0 with 3% acetic acid. When the solution was stirred strongly, myosin precipitated; this was separated by centrifuging, and dissolved by addition of KCl to a final concentration of 0.4–0.6 M. The purity of the preparation of myosin was tested by determining the viscosity of the myosin solution in an Ostwald viscosimeter, as it reacted with ATP. If the viscosity of myosin did not change after addition of the ATP, the preparation was considered pure.

TABLE 1

ATP-ase Activity of Myosin before and after the Action of High Pressure

Before pressure		After pressure	
μg P/mg N (5 min)	Q_p	μg P/mg N	Q_p
600	840	0	0
830	1160	0	0
1560	2160	0	0
800	1110	0	0

Obtaining myosin free from ATP-ase activity, but retaining the ability to dissolve in 0.6 KCl. The myosin was submitted to the action of high pressure (4000 atm) in a Bresler bomb [11] for 15 min. The resulting solution was dialyzed against twice-distilled water. After the precipitate which formed had settled, it was separated by centrifuging and dissolved in 0.6 M KCl. From the resulting solution myosin could again be precipitated by repeating the dialysis.

Obtaining actin. Actin was prepared by the method of Straube. Activated actin was produced by adding the salts: KCl to a final concentration of 0.6 M and $MgCl_2$ to a concentration of 0.0005 M.

Obtaining actomyosin. A sample of rabbit skeletal muscle was cut up with scissors in a cooled mortar. The resulting muscle mince was treated with 10 times its volume of Weber solution and stood in the cold for a day with periodic shaking. The resulting extract was separated from the pieces of tissue by centrifuging and diluted with 20 times its volume of cold twice-distilled water; the pH of the solution was brought to 6.8 with 3% CH_3COOH. After 3–4 hours the precipitated actomyosin was separated by centrifuging and dissolved by adding KCl to 0.6 M.

Determining the ATP-ase activity. The ATP-ase activity of the proteins was determined by the increase in inorganic phosphorus in a sample and was expressed by the value of Q_p. Composition of the sample: 1 ml of protein solution which contained 0.04–0.07 mg of nitrogen, 1 ml of borate buffer, pH 8.6, 0.5 ml of 0.03 M $CaCl_2$, 1 ml of 0.5% solution of ATP. The sample was incubated at 37° for 5 min. Reaction was stopped by adding 0.5 ml of 20% CCl_3COOH. Inorganic phosphorus was determined photometrically in the filtrate by the Fiske and SubbaRow method. In the control samples, trichloroacetic acid was added directly to the protein solution.

Obtaining actomyosin fibers free from ATP-ase activity. Rabbit skeletal muscle was cut up and extracted in the cold with Weber solution (3 ml per g of tissue) for a day. The muscle mince was passed through two layers of gauze. The resulting extract was diluted with 1.5–2 times its volume of Weber solution and separated by centrifuging. The centrifugate, actomyosin, was submitted to the action of high pressure (4000 atm) for 15 min. By the usual process (blowing in Szent-Györgyi solution) actomyosin fibers were prepared from this solution. The ability of the fibers to react with ATP (1% solution) was tested under the microscope with low magnification.

RESULTS

In solutions of reprecipitated myosin (before and after the action of pressure on it) we determined the ATP-ase activity. Table 1 shows that myosin has ATP-ase activity which is expressed by Q_p 800-2100.* The myosin treated by high pressure completely loses ATP-ase activity and its Q_p falls to zero.

TABLE 2

Change in Relative Viscosity of Myosin Solutions (Before and After the Action of High Pressure) on Added Actin and ATP
Results given for one of the experiments of this series

Before pressure				After pressure			
Myosin	Actin	Myosin + actin (3:1)	After addition of ATP	Myosin	Actin	Myosin + actin (3:1)	After addition of ATP
3.87	2.45	10.15	3.54	8.52	2.45	5.66	5.66

In this connection, there was certainly interest in discovering if the myosin preparation submitted to pressure maintained its ability to form a complex with actin. For this purpose we ran the following experiments. To a myosin solution (before and after the action of pressure on it) we added actin with a solution ratio of 3:1. We judged the formation of actomyosin by the sharp increase in the reaction of the "artificial" actomyosin which formed with ATP. The results of this series of experiments are given in Table 2.

The Table shows that myosin, before it is acted on by pressure, reacts with actin to form actomyosin. The relative viscosity of the initial myosin solution is increased, from 3.87 to 10.15. After addition of ATP, the viscosity of this "artificial" actomyosin falls to 3.54, which indicates its dissociation in the presence of ATP.

TABLE 3

Change in Relative Viscosity of Actomyosin Solutions (Before and After Action of High Pressure) when ATP Is Added
In the Table the results of one experiment of this series are given

Before pressure		After pressure	
Actomyosin	Actomyosin + ATP	Actomyosin	Actomyosin + ATP
9.44	3.77	9.44	8.66

* For the determination of ATP-ase activity of myosin we used an ATP preparation with a large ATP content, which, according to the data of Baev [12], results in a considerable lowering of Q_p in the determination of the enzymatic activity of myosin.

After the action of high pressure, myosin loses the ability to form a complex with actin, forming actomyosin: the viscosity of the mixture of myosin and actin in this case not only does not increase compared to the viscosity of the starting solution, but takes on an average value whose size is determined only by the initial viscosity and the volume of solution added. After addition of ATP to the mixture of actin and myosin treated with high pressure, no fall in viscosity is observed.

These data allow us to presume that under the influence of high pressure there occurs some change in the structure of the protein which is connected with the inactivation of some group (probably SH) which gives myosin the character of an enzyme, but is not accompanied, for myosin, by the loss of its hydrophilic properties or ability to dissolve in salt solutions with ionic strength of 0.35–0.4.

It is also interesting that, in agreement with the data of Poglazov, Bilushi, and Baev [13] who studied the changes of heat-denatured myosin, solutions of purified myosin under the influence of high pressure greatly increase their relative viscosity (in the case shown in Table 2, from 3.87 to 8.52).

Actomyosin is also inactivated under the influence of high pressure. Table 3 shows that actomyosin not subjected to the action of pressure has a strongly lowered relative viscosity under the influence of ATP (in this case, from 9.44 to 3.77). After the action of high pressure, actomyosin almost loses this ability. Thus, in the example given, the viscosity of a solution of actomyosin submitted to the action of pressure falls from 9.44 to 8.66 when ATP is added; that is, is almost unchanged, or, more accurately, is changed in the way which might be expected from simple dilution of the actomyosin. In a number of cases, the change in viscosity after addition of ATP is not found at all.

As to changes in relative viscosity of actomyosin in the absence of ATP after the action of high pressure, either there is no change (as in the case given), or there is a slight fall. However, this is not to be explained by the absence of dissociation of the actomyosin complex into its components (actin and myosin), but results from the action of the high pressure on the much more viscous myosin (possibly partly denatured). The latter can mask the fall in viscosity of the actomyosin complex as a result of its dissociation. This is also the reason why the viscosity of insufficiently purified myosin which still contains actomyosin does not increase as much after the action of high pressure as does that of the pure myosin preparation. (In Tables 2 and 3 we give the data for one of the typical experiments of each series).

We can prepare fibers from actomyosin gel acted on by high pressure, but these fibers do not react by contracting when ATP is added. Fibers obtained from the same actomyosin gel which has not been submitted to the action of pressure contract 2–2.5 times in the presence of ATP. It has also been established that muscle fibers soaked in 50% glycerol after the action of pressure lose the ability to contract when ATP is added in Szent-Györgyi solution.

All the above findings sharply contradict the data of Buchthal and co-workers [8] on the maintenance of contractile properties of muscle proteins when there is full inactivation of their ATP-ase activity.

SUMMARY

Solutions of reprecipitated myosin under the influence of high pressure (4000 atm) fully lose their ATP-ase activity, but retain their ability to dissolve well in 0.6 M KCl and to precipitate with later dialysis or strong dilution of the salt solution of myosin by water. The viscosity of myosin which does not contain contaminating actomyosin increases under the influence of high pressure. Myosin submitted to the action of high pressure loses the ability to react with actin to form actomyosin.

Actomyosin gel submitted to the action of high pressure (4000 atm) loses the ability to lower its viscosity when ATP is added. Actomyosin fibers obtained from actomyosin gels acted on by high pressure lose the ability to contract in the presence of ATP. Soaking muscle fibers in 50% glycerol after the action of high pressure also destroys their ability to contract in the Szent-Györgyi solution when ATP is added.

Thus the results of Buchthal and co-workers [8] on the ability of actomyosin gel, apparently deprived of ATP-ase activity, to undergo syneresis under the influence of ATP must be considered incorrect.

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A STUDY OF POLYPHOSPATE METABOLISM IN DEEP CULTURES OF PENICILLIUM CHRYSOGENUM Q-176

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In our previous studies [1, 2] we examined the changes which occur among polyphosphates and some other phosphorus compounds in surface cultures of Aspergillus niger.

Data similar to ours were also obtained by Krischnan and coworkers [3] who studied the polyphosphate metabolism in A. niger in both surface and deep cultures. It was specifically shown in this work that the polyphosphate metabolism among mold fungi is not materially changed by a change in the conditions of their cultivation. As to Penicillium chrysogenum Q-176, a considerable amount of polyphosphates was detected during its growth in a synthetic medium [4, 5], while the polyphosphates were not detected under cultivation in a medium containing corn extract in its composition [6].

The present work was set up with the aim of a further refining of the problem of the effect of the conditions of cultivation and, specifically, the nutrient-medium composition, on the polyphosphate metabolism among mold fungi.

MATERIAL AND METHODS

Penicillium chrysogenum Q-176 was grown under sterile conditions of submerged growth at 24° in a liquid synthetic medium of Stone and Farell [7] without zinc, to which medium a small amount of potassium chloride (0.5 g/ liter) was also added. The pH of the medium was adjusted to 6.5. Corn extract was added, in the amount of 20 g of dry weight per liter, in a number of cases to this medium. For securing a sufficient amount of spores necessary for both the inoculation and for the chemical analysis, P. chrysogenum was grown in the same nutrient medium without the corn extract under the conditions of a surface culture growth. The 7-day mycelium was used for spore collection. In addition to the spores, the material of the following stage of development of P. chrysogenum was also used for the chemical fractionation: in a medium without the corn extract – 12, 18, 24, 31, 36, 48, 60 and 96 hours of growth. The collected material was thoroughly washed free of the medium elements by means of water and was fixed with alcohol. The material fixed in this manner was then dried lyophilically.

It was established in a series of control experiments that the acid-soluble polypnosphates, usually extracted with 5% trichloroacetic acid (TCA) in the cold [1, 3], may be separated by the Lohmann and Langen method [8] into two fractions which include in their composition the polyphosphates with different chain lengths.

As is evident from the cited data (Table 1), the amount of labile phosphorus (P_l) and total phosphorus (P_{tot}) in the extract with cold 5% TCA corresponded sufficiently accurately to their amount detected in the fractions obtained by the Lohmann and Langen method (extract with 1% TCA, then salt-extract).

These data indicated the possibility of replacement of the generally accepted extraction method for acid-soluble phosphorus compounds by the Lohmann and Langen method. As is evident from Table 1, the extraction with hot 5% TCA, used for the extraction of acid-insoluble polyphosphates and nucleic acids (NA), may be replaced by extraction with hot 10% $HClO_4$, used in the work of Krischnan and co-workers [3]. This change allows for a spectrophotometric determination of the amount of NA which is impossible to realize in the TCA extracts.

TABLE 1

Results of Comparison of Extraction Methods of Acid-Soluble and Acid-Insoluble Polyphosphates from P. Chrysogenum Q-176

Data in μg per 1 g of dry weight of the mycelium

Form of phosphorus	Acid soluble fraction			Acid insoluble fraction	
	Extraction with cold 5% TCA	Subsequent extraction		Extraction with hot 5% TCA	Extraction with hot 10% $HClO_4$
		With cold 1% TCA	With saturated $NaClO_4$ solution		
Total P	14 566	10 020	4 520	8 970	9 238
Labile P	716	0	678	–	–

On the basis of these preliminary experiments we settled on the following fractionation scheme. The lyophilically dried material (400 mg) was extracted twice with 30 ml of 1% TCA at 0–2°: for 90 minutes the first time and for 15 minutes the second time. The precipitate was washed once with water and the wash water was combined with the extracts. The thus-obtained fraction contained the low molecular weight, acid-soluble polyphosphates. 1.5 g of $NaClO_4$ and 1 ml of 10% TCA were added to the residue after the removal of the lower polymers, and the thick suspension obtained as a result of this treatment, was set aside with stirring for 15 minutes in the cold. Then, 15 ml of water was added to this suspension and the extraction was continued for 15 minutes longer. This extraction was repeated twice. The residue after the extraction was washed several times with a small volume of water. The extracts were combined with the wash water. The relatively higher molecular-weight fragments of polyphosphates of the acid-soluble fraction entered the composition of the resulting salt extract.

The residue after the salt extraction was treated successively with alcohol, twice with a hot mixture of alcohol-ether (3:1) and, finally, with ether, for a complete removal of the phospholipids.

The residue was treated with 30 ml of 10% $HClO_4$ for 30 minutes in a steam bath for the extraction of acid insoluble polyphosphates. The extract was separated from the residue by centrifuging and the residue was again treated under the same conditions with 15 ml of 10% $HClO_4$ for 15 minutes. The residue after the second extraction was repeatedly washed with water, while the wash waters were combined with the extracts, as in the other cases.

The determination of various forms of phosphorus was run in the resulting fractions. Phosphorus was determined by the Bosse method [8] in all cases.

By means of this method we determined in the first two (acid-soluble) fractions: the inorganic phosphorus (P_i) directly in the extract, and phosphorus mineralized by 10 min hydrolysis on a steam bath for N HCl and P_{tot} after combustion with concentrated $HClO_4$. (P_i) was calculated by difference between the "10-minute" P and P_i. while the stable P was determined by difference between total and "10-minute" P. P_{tot}, in addition, was determined in the material, before treatment, in the acid-insoluble fraction, in the phospholipid fraction and in the residue after the removal of the acid-soluble and acid-insoluble fractions. In addition, ribonucleic acid was determined spectrophotometrically [9] in the acid-insoluble fraction along with P_i formed as the result of hydrolysis with 10% $HClO_4$, the magnitude of this being used for an approximate judgment of the amount of acid-insoluble polyphosphates. If only NA and polyphosphates were present in this fraction, the exact content of the latter was judged by the difference between total and nucleic P of the extract. At the same time, in mycelium grown in the medium with the corn extract, the exact calculation of polyphosphate P in this fraction proved to be impossible owing to the presence of other phosphorus compounds in it. The amount of polyphosphates in this fraction could be estimated only approximately from P_i formed as a result of hydrolysis with 10% $HClO_4$.

Total nitrogen was determined by the Kjeldahl method and was provisionally taken to be protein nitrogen, in the residue after the removal of all the fractions.

All data secured by the chemical analysis of the various fractions were expressed in μg per 1 g of dry weight of the material and per 100 ml of the medium, allowing for the amounts of substances which had passed into the alcohol-ether fraction.

RESULTS OF THE WORK

The data on the distribution of various forms of P in all the fractions obtained by us from P. chrysogenum Q-176 in the process of its development in a medium without the corn extract are given in Tables 2 and 3. The analytical data, calculated per 1 g of dry weight of the material (Table 2) indicate first of all that a very large amount of labile acid-soluble phosphorus, comprising at that moment about 40% of P_{tot} of the material, is contained in the spores prior to their being seeded onto the nutrient medium.

TABLE 2

Change of Some Phosphorus Compounds in the Process of Development of P. Chrysogenum Q-176 in a Medium Without the Corn Extract
In μg P per 1 g of dry weight

Duration of growth, in hours	Protein nitrogen, mg/g of dry substance	P_{tot} of the material	P_{tot} of alcohol-ether fraction	Acid soluble fraction							Acid insoluble fraction		
				Total ortho-phosphate	Extract with cold 1% TCA			Extract with cold saturated $NaClO_4$ solution			Extract with hot 10% $HClO_4$		Residue
					Ortho-phosphate	P_1	Stable phosphorus	Ortho-phosphate	P_1	Stable phosphorus	Polyphosphate phosphorus	Nucleic phosphorus	P_{tot}
0	15,31	16 150	214	2 810	2 790	4904	0	20	2183	476	444	2985	377
12	11,61	18 075	181	4 732	4 695	333	830	37	1283	462	2 491	5267	903
18	13,95	39 231	134	15 563	13 554	0	564	2009	72	28	12 053	8681	156
24	14,87	28 284	160	8 954	7 678	94	651	1276	1754	114	8 181	8766	20
31	22,92	26 150	260	5 392	5 132	206	361	260	2372	259	7 471	9752	70
36	35,17	24 092	174	5 554	5 152	0	1031	402	1422	580	6 816	7276	229
48	25,91	28 195	—	15 538	14 712	0	665	826	1549	996	5 323	3915	209
60	12,15	28 446	92	17 184	16 458	0	729	726	963	851	4 954	3167	515
96	7,89	17 652	101	10 840	10 324	0	663	516	367	26	3 085	2226	60

TABLE 3

Change of Some Phosphorus Compounds in the Process of Development of P. Chrysogenum Q-176 in a Medium Without the Corn Extract
In μg per 100 ml of the medium

Duration of growth, in hours	Total biomass in g/100 ml of medium	P_{tot} of the material	P_{tot} of alcohol-ether fraction	Acid soluble fraction							Acid insoluble fraction		
				Total ortho-phosphate	Extract with cold 1% TCA			Extract with cold saturated $NaClO_4$ solution			Extract with hot 10% $HClo_4$		Residue
					Ortho-phosphate	P_1	Stable phosphorus	Ortho-phosphate	P_1	Stable phosphorus	Polyphosphate phosphorus	Nucleic phosphorus	P_{tot}
0	0,0007	11,3	0,14	1,9	1,95	3,4	0	0,014	1,5	0,33	0,31	2,1	0,26
12	0,0007	12,7	0,13	3,3	3,29	0,23	0,58	0,026	0,9	0,32	1,7	3,7	0,63
18	0,0344	1349	4,6	535	466	0	19	69	2,5	1,0	415	299	5,4
24	0,0911	2577	15	816	699	8,3	59	116	160	13	445	799	2,0
31	0,1270	3527	34	685	652	26,1	46	33	301	33	949	1239	9,1
36	0,2729	6575	47	1516	1406	0	281	110	388	158	1860	1986	62
48	0,3662	9815	—	5690	5388	0	244	302	567	365	1949	1434	76
60	0,3663	10 682	34	6300	6034	0	267	267	353	312	1816	1161	189
96	0,5212	9179	52	5637	5368	0	345	268	190	14	1604	1158	31

It should be noted that P_l is contained in a large amount in both the salt and the 1% TCA extract of the spores. This indicates the existence of a rather large amount of low-polymeric polyphosphates, along with the high-polymeric ones, in the composition of the acid-soluble fraction of the spores.

During the first 12 hours of spore growth, as is evident from the data in Table 2, a very considerable reduction of the acid-soluble polyphosphates, especially in the low-polymeric fraction, occurs, the latter fraction being extractable with 1% TCA. During the same period a certain reduction of the protein nitrogen is observed, this occurring along with a rather considerable increase of the amount of acid-insoluble polyphosphates and NA.

During the further development of the fungi, i. e., during the period between 12 and 18 hours of growth, during which an intense entry of the nutrient elements of the medium into the young mycelium takes place, there is detected a very active synthesis of acid-insoluble polyphosphates and NA. The amount of P of acid-insoluble-polyphosphates toward the end of this period, as is evident from the data in Table 2, comprises about 30% of P_{tot} of the material. It is characteristic that toward this moment P_l of the acid-soluble fractions disappears almost completely.

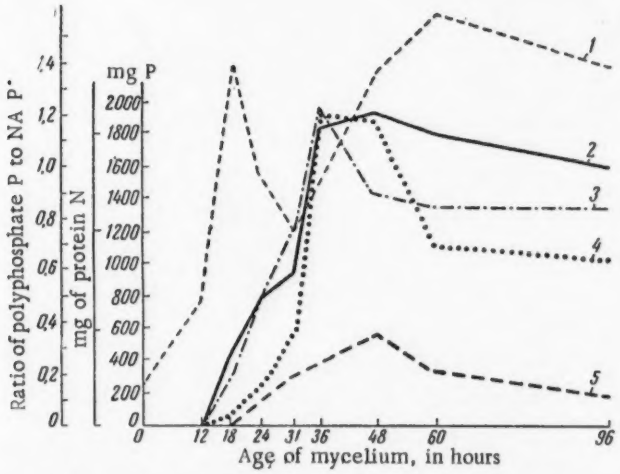

Fig. 1. Change of content of polyphosphates, nucleic acids and protein in the process of development of P. chrysogenum Q-176 in a medium without corn extract (calculations based on 100 ml of medium). 1) Ratio of phosphorus in acid-insoluble polyphosphates to nucleic acid phosphorus; 2) phosphorus of acid-insoluble polyphosphates; 3) nucleic acid phosphorus; 4) protein nitrogen; 5) phosphorus of acid-soluble polyphosphates.

It follows from the consideration of the data in Table 3, calculated on 100 ml of the medium and giving a true picture of the intensity of the synthesis of various compounds in the cells, that the accumulation of the acid-insoluble polyphosphates during this period takes place at a considerably greater rate than the nucleic acid synthesis. The synthesis of nucleic acids occurs at a considerably greater rate than the accumulation of polyphosphates during the following development period of the fungus. The synthesis of acid-insoluble polyphosphates precedes the intense formation of NA in which the polyphosphate fraction evidently takes a direct part. This conclusion obviously follows from the graphic representation of the changes in the polyphosphate content during the period of development of the fungus (Fig. 1). It follows from the consideration of the data shown in Fig. 1, that the ratio of the amount of phosphorus in acid-insoluble polyphosphates to the NA phosphorus drops sharply during the period of the most intense NA synthesis. The decrease of this value on the background of a rather energetic synthesis of polyphosphates of the acid-insoluble fraction may indicate, indirectly, their utilization in the NA synthesis. An intense protein synthesis begins in the mycelium only after a rather considerable amount of NA has formed in it. With the beginning of active biosynthesis of protein, the formation of the acid-insoluble polyphosphates is again strengthened, which fact favors a direct utilization of polyphosphates in this synthetic process, as well.

A very strong consumption of the orthophosphate occurs during the period of the most energetic synthetic processes (Table 2). The amount of orthophosphate in the mycelium drops considerably, despite its still rather intense uptake from the medium (Table 3). It is evident from the consideration of the data in Table 2 that, by 24 hours of growth, the acid-soluble polyphosphates appear again in the mycelium, which is still actively synthesizing protein and nucleic acids; this occurs alongside a sharp drop in the acid-insoluble polyphosphate content. This is indicated by the detection of P_l during this period of development of the fungus in the fraction which is extracted by saturated $NaClO_4$ solution. A certain amount of P_l also appears in the 1% TCA extract during this time interval, but this P_l may be related not to polyphosphates but to some other labile phosphorus compounds of the TCA fraction [8].

The process of degradation of the acid-insoluble polyphosphates is continued with the parallel formation of acid-soluble polyphosphates as is evident from the data in Fig. 1, even during the period of 36 to 48 hours of growth, which is already characterized by the rather considerable weakening of the processes of protein biosynthesis and NA biosynthesis. During this period of life of P. chrysogenum (Table 3) there is observed the disappearance of P_l in the fraction extractable by 1% TCA, along with the further accumulation of polyphosphates in the salt extract. In the following period of development of the fungus (from 48 to 96 hours of growth), and despite the continuing increase of the total biomass of the mycelium, (Table 3), a further blocking of synthesis of NA and protein occurs (Fig. 1) in it. Simultaneously, a further degradation of the polyphosphates occurs in the mycelium, which touches upon not only the acid-insoluble polyphosphate fraction, but also the less-polymeric, acid-soluble fraction, as well (Table 2).

TABLE 4

Change in some Phosphorus Compounds in the Process of Development of P. Chrysogenum Q-176 on a Medium With Corn Extract
(In μg per 1 g of dry weight)

Duration of growth in hours	P_{tot} of the material	P_{tot} of alcohol-ether fraction	Acid soluble fraction							Acid insoluble fraction			
			Total orthophosphate	Extract with cold 1% TCA			Extract with cold saturated $NaClO_4$ solution			Extract with hot 10% $HClO_4$			Residue
				Orthophosphate	P_l	Stable phosphorus	Orthophosphate	P_l	Stable phosphorus	Polyphosphate phosphorus	Nucleic phosphorus	Stable nonnucleic phosphorus	P_{tot}
0	16 150	214	2810	2790	4904	0	20	2183	476	444	2985	0	377
14	—	—	3788	3765	1029	—	23	95	—	1333	6262	—	—
18	49 877	444	3911	3892	552	23 758	19	760	6031	2048	8433	4351	63
24	44 272	93	3865	3839	1070	25 428	26	1006	4704	1963	7011	1914	70
31	40 627	241	4322	4141	490	—	181	1449	4577	1707	6428	1934	85
48	42 682	181	4592	4556	506	28 691	36	945	5994	794	5063	2636	100
96	—	—	23 232	22 814	0	—	418	392	—	0	2993	—	—

It is impossible to detect even traces of P_l (Table 2), during this period of development of the fungus, in the 1% TCA extract, which fact indicates the absence of low-polymeric polyphosphates in the mycelium. Thus, the resulting data indicate that with the submerged cultivation of P. chrysogenum the degradation of the relatively high-polymeric polyphosphates of the acid-soluble fraction does not proceed through the stage of the lower polymers extractable by 1% TCA, but directly through the orthophosphate formation.

It is interesting that the considerable amount of P_l in the 1% TCA extract is detected in the unsprouted spores. In order to explain whether or not the high level of P_l in the spores is connected with the presence in them of large amount of low-polymeric polyphosphates, we isolated from the strongly spore-bearing 7-day mycelium of P. chrysogenum grown under the conditions of surface growth, the fractions of low-polymeric and high-polymeric acid-soluble polyphosphates by the method of Lohmann and Langen [8] and subjected these to a chromatographic analysis.

The chromatographic separation of the polyphosphates was run in the cold with the aid of the acid developer suggested by Grunze and Thilo [10] on Schleicher & Schul No 2943 paper in Mg, previously washed with 2N HCl for 36 hours. The ascending method of chromatography was used. The chromatograms were developed by the method of Hanes and Ischerwood [11].

It should be noted in the examination of the resulting chromatogram (Fig. 2) that in the case of a specimen of high-polymeric polyphosphates, all phosphorus remains at the point of deposition, which fact indicates the rather high level of polymerization of polyphosphates which compose it. A rather large amount of phosphorus remains also at the point of deposition during the chromatography of the low-polymeric polyphosphate fraction, which indicates the presence of relatively high-polymeric polyphosphates in this specimen as well, the latter being immobile under the given conditions of chromatographic separation. In addition to the above-cited relatively high-polymeric fragments, a rather considerable amount of ortho- and pyrophosphate enters the composition of the low-polymeric specimen. The presence of, specifically, pyrophosphate in the preparation, and not that of some other compound, such as fructose-1,6-diphosphate (F-1,6-P) which locates itself in approximately the same region on a chromatogram under these conditions, was proven by specially set-up experiments. These experiments show that with a complete disappearance of F-1,6-P and other phosphoric esters of sugars from the chromatogram after the treatment of the latter with ammonia vapor, the synthetic pyrophosphate and the phosphorus compound located at its level during the chromatography of a specimen of low-polymeric polyphosphates are not decolorized, which indicates the presence of the pyrophosphate itself.

It should be indicated that pyrophosphate was detected in the mycelium of A. niger, along with the polyphosphate, by Mann [12] as long ago as 1944, although no considerable amount of this compound has been found up to the present in the yeast organisms [8].

TABLE 5

Change of some Phosphorus Compounds in the Process of Development of P. Chrysogenum Q-176 on a Medium With Corn Extract
In μg per 100 ml of the medium

Duration of growth, in hours	Total biomass, g/100 ml of the medium	P_{tot} of the material	P_{tot} of alcohol-ether fraction	Acid soluble fraction						Acid insoluble fraction			
				Extract with cold 1% TCA			Extract with cold saturated $NaClO_4$ solution			Extract with hot 10% $HClO_4$			Residue
				Ortho-phosphate	P_l	Stable phosphorus	Ortho-phosphate	P_l	Stable phosphorus	Polyphosphate phosphorus	Nucleic phosphorus	Stable non-nucleic phosphorus	P_{tot}
0	0,0007	11,3	0,14	1,95	3,4	0	0,014	1,5	0,33	0,31	2,1	0	0,26
14	0,0213	—	—	80,2	21,9	—	0,50	2,0	—	24,4	135	—	—
18	0,0378	1885	16,8	147	20,8	898	0,71	29	228	77,4	319	164	2,3
24	0,3753	16 615	34,9	1440	401	9543	9,8	377	1765	737	2631	718	26,2
31	0,4509	18 282	108,5	1863	290	—	81	652	2060	768	2892	870	38
48	0,7749	38 360	140,3	3530	392	22 233	28	732	4644	615	3923	2043	77
96	1,1254	—	—	25665	0	—	470	442	—	0	3367	—	—

After this, we grew P. Chrysogenum Q-176 on the same synthetic medium of Stone and Farell but with the addition of a certain amount of corn extract, usually added to the medium in penicillin production. It is clear from the analytical data (Tables 4 and 5) that even under these conditions a considerable amount of P_l is detected in all fractions at all development stages of the fungus, except that of old mycelium (96 hours of growth). The presence of P_l in the salt fraction as well as in the fraction extractable by hot 10% $HClO_4$ undoubtedly indicates the presence of polyphosphates in this mycelium. It is also evident that the character of the changes in the polyphosphates during the development process of P. chrysogenum in a medium with the corn extract does not differ in the main in any way from that obtained in the growth of the fungus on the usual synthetic medium (Table 2). The amount of acid-soluble polyphosphates decreases greatly and the amount of acid-insoluble polyphosphates increases during the process of sprouting of the spores on a medium with the corn extract, as well as on the usual medium. The content of physiologically active acid-insoluble polyphosphates in a young, actively synthesizing mycelium (18 and 24 hours of growth) is maximum (Table 4), while the content of acid-soluble polyphosphates is relatively not great but, as shown by the data in Table 5, these gradually begin to accumulate with the weakening of the most important synthetic processes, specifically, synthesis of NA. With the further fungus development

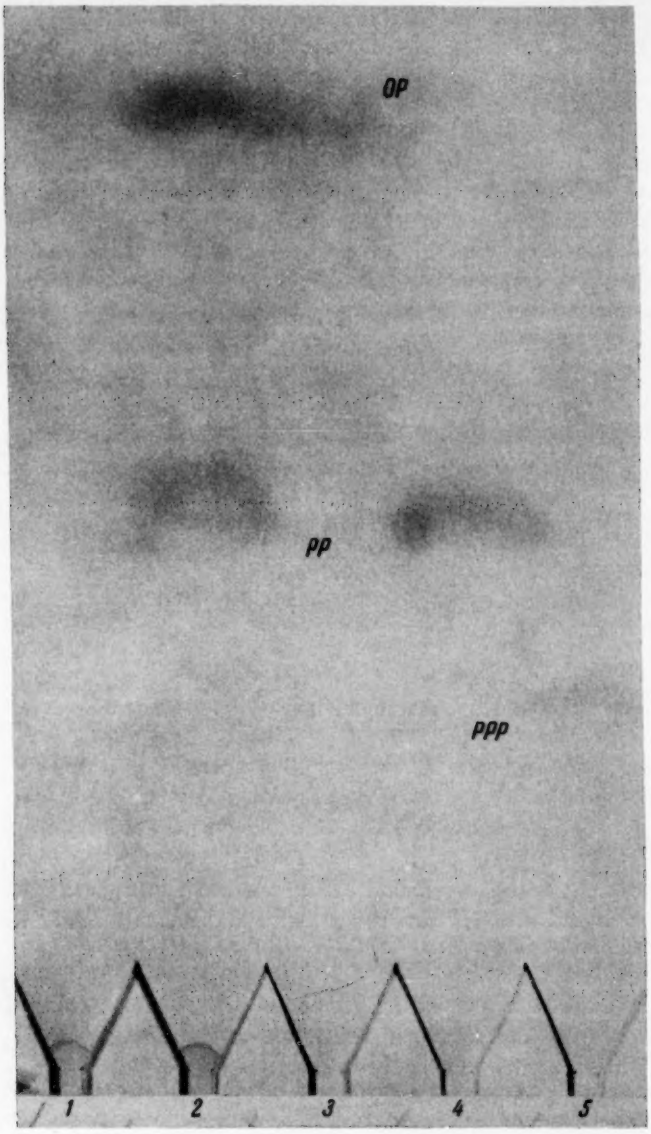

Fig. 2. Chromatogram of low-polymer and high-polymer fraction of acid-soluble polyphosphates from spore bearing mycelia of P. chrysogenum Q-176:
1) high-polymer fraction; 2) low-polymer fraction; 3) orthophosphate; 4) pyrophosphate; 5) tripolyphosphate

(Table 4), more and more of the acid-soluble polyphosphates are present in the mycelium in comparison with the acid-insoluble ones. This leads to the situation in which the 96-hour mycelium has still detectable amounts of the acid-soluble polyphosphates with a total absence of the acid-insoluble polyphosphates in the salt fraction.

It should be pointed out that the ratio of acid-soluble and acid-insoluble polyphosphates in the P. Chrysogenum mycelium, cultivated on a medium with the corn extract, is shifted in comparison with that in the usually cultivated fungus, the shift being toward the acid-soluble polyphosphates (Tables 2 and 4). The total level of

polyphosphate content in the fungus grown on a medium with the corn extract is considerable lower that found in cultivation on the usual synthetic medium (Tables 2 and 4). The intensity of polyphosphate synthesis, as indicated by the calculations, per 100 ml of the medium (Tables 3 and 5) during the fungus development on the medium with corn extract is considerably lower than under the conditions of the normal nutrient medium. Growing of P. chrysogenum on a medium with the corn extract is manifested very favorably in the growth and development of its mycelium, which is confirmed by the very great intensity of accumulation of the total biomass of the mycelium (Table 5). Such a high level of accumulation of the biomass of the mycelium during the fungus growth on a medium with the corn extract is very well correlated with the enhancement of the rate of NA biosynthesis which occurs under these conditions. The maximum content of NA in this case is reached considerably later and represents a magnitude which is two times greater than in the mycelium grown under the usual conditions (Tables 3 and 5).

When we consider the resulting data, we get the impression that the improvement in growth and development of the fungus leads to a lowered level of polyphosphate synthesis in it. We should remark that Krishpan and co-workers [3] recently came to the same conclusion with respect to A. Niger.

An impression is gained during the examination of the data in Table 4 that, despite the relatively low content of labile phosphorus compounds in the mycelium grown on a medium with the corn extract, the total amount of phosphorus is almost two times higher than it is in the mycelium of corresponding age that was cultivated on the usual nutrient medium. Such high values of total phosphorus are connected with the accumulation in the mycelium of rather large amounts of some stable phosphorus compounds. An especially large amount of the stable phosphorus is discovered in the 1% TCA extract; more than 50% of total phosphorus of the mycelium falls to its share in a number of cases. We established that the stable phosphorus of this fraction is precipitated by the magnesia mixture and, thus, may be classed as the phytin phosphorus. In addition to the 1% TCA extract, a rather large amount of stable phosphorus was discovered in the salt fraction and in the fraction extractable with hot 10% $HClO_4$ (Table 4). These phosphorus compounds are not precipitated by the magnesia mixture and are as yet unidentified.

DISCUSSION OF THE RESULTS

Essentially the same regularities which had been obtained previously in connection with the surface-developed A.niger [1, 2] were disclosed in the study of the polyphosphate metabolism in P. chrysogenum Q-176 in its deep (submerged) cultures. This agrees with the data from the work of Krischnan and co-workers[3], who had also shown that any material differences are absent between the polyphosphate metabolism in surface- and submerged-grown mold fungi. The results of the present work confirm that the acid-insoluble polyphosphates possess the greatest physiological activity; these polyphosphates, in all probability, participate in the direct manner in the synthesis of nucleic acids and in the protein synthesis (Fig. 1). We showed recently that the acid-insoluble polyphosphates may be connected with ribonucleic acid in the cell (ribonucleic acid – RNA) [13]. In this connection one should recall the hypothesis expressed as long ago as 1946 by Spiegelmann and Kamen [14] concerning the RNA, phosphorylated by polyphosphates, as being the specific donor of energy and phosphorus in protein biosynthesis. This view is upheld by some other authors [15].

The resulting data confirm the previously advanced and experimentally founded [1-3, 16-18] view that the synthesis of polyphosphates occurs in the acid-insoluble fraction. Along with this, the acid-soluble polyphosphates are formed secondarily by the way of degradation of the acid-insoluble ones.

The process of degradation of acid-insoluble polyphosphates to the lower-polymeric ones – acid-soluble ones – proceeds differently in the surface and the deep cultures.

During the surface growth of the mold fungi under conditions of spore formation, the acid-insoluble polyphosphates probably decompose gradually with formation of, at first, high-polymeric acid-soluble polyphosphates, then low-polymeric ones and, finally, orthophosphate. In a strongly spore-bearing mycelium, a rather large amount of the pyrophosphate is present along with the high-polymeric polyphosphates in the acid-soluble fraction. It is possible that the transport of phosphorus and energy, accumulated in the polyphosphate, from the mycelium into the spores takes place specifically in the form of the pyrophosphate.

Under the conditions of the submerged growth of the mold fungi, the low polymers of polyphosphates are totally absent from the mycelium and the degradation of the high-polymeric fragments proceeds by the way of a direct formation of orthophosphate. It is interesting to note that Kischnan [3] failed to isolate any pyrophosphate from a submerged-growing mycelium of A. niger. Thus, the resulting data indicate that the process of degradation of poly-

phosphates may be changed, depending on the conditions of cultivation of the organism, although the general character of polyphosphate metabolism is unchanged in principle with alteration of the conditions of growth as well as with different compositions of the nutrient medium.

Growing of P. chrysogenum in a medium with the corn extract, in lowering the total level of polyphosphate content in the mycelium, does not at all affect the fundamental regularities of polyphosphate metabolism in the process of development of this organism. The fact of the detection of polyphosphates in P. chrysogenum during its cultivation in a medium with the corn extract goes contrary to the data from the work of Gotovtseva [6] who failed to find any appreciable amount of polyphosphates in this organism under similar growth conditions. The contradiction is evidently explained by the difference in the age of the analyzed mycelium. Gotovtseva used only four-six-day mycelia, in which the polyphosphates may be completely absent under these conditions of cultivation. In a 4-day mycelium, grown in a medium with the corn extract, we succeeded in detecting the polyphosphates only in the salt extract and, even there, in but a very small amount (Table 4).

It is necessary to note that a large amount of some stable phosphorus compounds is accumulated during the growing of P. chrysogenum on a medium with the corn extract. In all probability, the stable phosphorus in the 1% TCA extract in this instance is the phytin phosphorus, as proven by its precipitation by the magnesia mixture and by the fact that Gotovtseva [6] in growing P. chrysogenum in a medium with the corn extract discovered a large amount of this phosphorus compound in the acid-soluble fraction and later precisely identified this substance [19]. As to the stable phosphorus compounds of the salt extract and of the fraction extractable by hot 10% $HClO_4$, nothing definite can be said about them at this time. It is not exluded that they are phosphorylated polysaccharides similar to those discovered by Belozerskii and Naumova [20] among the actinomycetes.

It is quite possible that the lower level of the content of polyphosphates encountered in the growing of P. chrysogenum in a medium with the corn extract may be explained by the fact that the phosphorus which enters the mycelium is used not for the polyphosphate synthesis but is utilized for formation of these stable phosphorus compounds.

SUMMARY

It was shown that in deep growing (submerged culture) of mold fungi P. chrysogenum Q-176, the polyphosphate metabolism is accomplished under the same rules as are shown in the surface growth (cultivation) of these organisms.

Some differences in the polyphosphate metabolism in deep and surface growth of the mold fungi evidently exist only in the process of degradation of the high-polymeric acid-soluble polyphosphates. In deep cultivation of P. chrysogenum the polyphosphate fraction is depolymerized by the way of direct formation of orthophosphate. In surface growth of the mold fungi under the conditions of spore formation, the degradation of the high-polymeric-acid soluble polyphosphates proceeds, in all probability, in such a manner that relatively low molecular-weight fragments of polyphosphates are formed first, and these are then subjected to a further depolymerization with formation of orthophosphate. The pyrophosphate is evidently the intermediate stage of degradation of the polyphosphates under the conditions of surface growth of P. chrysogenum and it may be in the form of this pyrophosphate that the transfer of phosphorus and energy from the mycelium to the spores takes place.

A considerable amount of polyphosphates was detected in growing of P. chrysogenum in a medium with corn extract, the alteration of these polyphosphates in the process of development of the fungus having the same character as found in its cultivation in the usual synthetic medium.

The polyphosphate content in mycelium of P. chrysogenum is relatively small during its growth in a medium with the corn extract.

A great part of phosphorus, detected in the mycelium in its cultivation in a medium with the corn extract, is in the portion composed of some stable phosphorus compounds. In all probability, phytin and some other (both acid-soluble and acid-insoluble) phosphorus compounds of unknown nature enter its composition.

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SENSITIVITY OF PROTEINS OF THE BONE MARROW OF IRRADIATED ANIMALS TO PROTEOLYTIC ENZYMES

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It was shown in our previous papers [1, 2] that after a massive irradiation of rabbits (1000 r), the intensity of autolysis of the bone-marrow tissue is considerably increased. The change in autolysis may be observed from the first minutes after irradiation, and the maximum rise occurred after 24 hours following the action of ionizing radiation. An analysis of the possible causes of enhancement of autolysis led us, firstly, to study the cathepsin activity in this tissue before and after irradiation. However, the experiments run in this direction failed to show an increase of activity of the cathepsins of bone marrow during the period of the highest intensity of autolysis, when studied by Anson's method [3]. One of the probable mechanisms of enhancement of the process of self-digestion may also be the alterations among proteins themselves which arose as a result of irradiation and which assured a more ready accessibility of these for the action of proteolytic enzymes. In the present communication we give the materials from a study of the enzymic cleavability of proteins of bone marrow by the action of pepsin and trypsin.

METHODS OF STUDY

The experiments were run on 36 rabbits, weighing 2.5–3.0 kg. The animals were irradiated with γ-rays from Co^{60}. Dose: 1000 r, dose intensity: 15 r/ min. The proteolysis of bone-marrow proteins was studied after 10 min, 4, 12 and 24 hours and 13 days following the irradiation. The cleavability of proteins of control, unirradiated animals was studied simultaneously. In all, 9 control and 27 experimental rabbits were studied. The animals were killed by air embolism.

The rate of proteolysis was determined by the amount of tyrosine (and tryptophan) liberated after the action of pepsin and trypsin from the tissue proteins of bone marrow. Commercial specimens of pepsin and trypsin were used for this work. It is possible that the trypsin contained a certain amount of chymotrypsin. Experiments with pepsin were run in 0.01N HCl at pH 2.0; those with trypsin, in phosphate buffer at pH 8.0. A 15% aqueous tissue homogenate from bone marrow was used as the substrate, this being added to the sample in 1 ml volume. The samples were incubated at 37° for 15 and 30 minutes. After precipitation of the proteins, the amount of tyrosine and tryptophan was determined in the filtrate by means of Folin reagent [4]. The results are given in micrograms of tyrosine liberated from the bone-marrow tissue proteins per 1 mg of nitrogen in this tissue. The spontaneously cleaved tyrosine, determined in parallel samples without the addition of enzymes, was subtracted from the amount of tyrosine discovered in the samples with the added proteolytic enzymes. The nitrogen was determined by micro Kjeldahl method.

RESULTS AND DISCUSSION

The data on the study of the rate of proteolysis of proteins of the bone marrow of control and irradiated animals are given in the table, from which it is evident that the rate of cleavage of tyrosine in the irradiated animals is considerably higher than in the control, unirradiated rabbits. The definite increase of tyrosine cleavage was already observed after 10 min following the end of irradiation. With 15 minute incubation, the activity of proteolysis in samples with pepsin and trypsin was raised noticeably and amounted to 139 and 155% of the corresponding data from the unirradiated animals. The intensity of proteolysis remained at the same level after 4 hours following the irradiation and rose somewhat after 12 hours. This rise was most clearly displayed in samples

with trypsin and amounted to 210% in comparison with the norm (15-minute incubation). The rate of cleavage of tyrosine declined somewhat after one day and this was especially noticeable in the analysis of the data on proteolysis with trypsin. It was possible to observe a further acceleration of proteolysis after six days (about 200% of the corresponding norm) which in samples with trypsin continued even after 13 days (267%). A statistical check of the difference of arithmetic means of the control and the experimental groups (Student method) proved its validity in most of the cases for 15-minute incubations when the highest rate of tyrosine cleavage took place. Also valid were the differences between the first periods, 24 hours and 13 days (Table).

Intensity of Proteolysis of Proteins of Bone Marrow at Various Intervals After Irradiation (Dose 1000 r)

Time after irradiation	Number of experiments	Amount of tyrosine liberated, μg/mg N — Incubation at 37° for		p***	
		15 min	30 min	15 min	30 min
Action of pepsin					
Norm	9(9)*	118±13**	135±14	—	—
10 min	3(4)	164±21	158±16	0,10	0,40
4 hours	3(3)	158±16	166±12	<0,10	>0,10
12 »	3(5)	179±13	215±12	0,01	0,10
24 days	3(3)	167±15	192± 6	<0,05	<0,01
6 days	4(5)	231±25	255± 4	<0,05	<0,001
13 »	5(7)	229±29	254±26	0,05	<0,001
Action of trypsin					
Norm	9(9)	71±10	88± 7	—	—
10 min	3(4)	110±13	115±22	<0,05	>0,2
4 hours	3(3)	107± 5	118± 0	<0,05	0,002
12 »	3(5)	152± 7	165± 6	<0,001	0,001
24 »	3(3)	111±12	118±12	<0,05	0,05
6 days	4(5)	151±20	174±20	0,005	0,002
13 »	5(7)	190±25	201±25	0,001	0,001

* The bone marrow from two rabbits was studied simultaneously at certain time intervals. The number of animals studied in each time intervals is shown in parentheses.

** An error in arithmetic mean

*** The probability that the difference between the mean values of control and experimental groups is accidental.

Thus, the cited experimental data confirmed the supposition made by us previously that the enzymic cleavability of bone-marrow proteins rises after irradiation. The absence of direct data about the alteration of the structure of proteins of bone marrow after irradiation does not permit us to make any definite conclusions about the nature of the changes in the protein molecule discovered by us.

In the analysis of the probable mechanisms responsible for the change of the rate of proteolysis in the proteins of the irradiated organism we turned our attention, first of all, to the denaturation phenomenon.

It is known that the sensitivity of denatured proteins in respect to proteolytic enzymes is a material criterion of denaturation [5]. The investigators connect the enhanced sensitivity of denatured proteins to the action of enzymes with the fact that the inner peptide links become accessible to enzyme action as a result of this process. It is possible that under the conditions of our experiments, the products of radiolysis of water, the excitation and, possibly, to some degree, direct ionization of the protein molecules themselves lead only to the rupture of the weak intramolecular bonds. The rupture of the peptide bonds seems to us as less probable, on the basis of data from the work of Volkova and Pasynskii [6] as well as those of Pasynskii and co-workers[7], secured after irradiation of solutions of serum albumin by ultraviolet and X-rays. Evidently, the phenomena of mild denaturation already arise

in the protein of bone-marrow cells during the process of irradiation, and grow with time; and it is specifically these phenomena that determine the enhanced sensitivity of proteins to pepsin and trypsin in the first intervals of the study.

The closest to our observations are the data from the work of Épshtein and Zabozlaeva [8, 9] who also discovered a more rapid cleavage of the irradiated serum albumin by pepsin. The effect of acceleration of peptolysis was observed by them in vitro after irradiation of dilute solutions of albumin and in vivo on the albumin, isolated from the blood serum of dogs, after administration to them of toxic doses of radioactive iron.

It is interesting that the labilization of the link between protein and DNA in the molecule of desoxynucleoprotein, isolated from the spleen of irradiated animals, in respect to the action of trypsin, has been recently shown by Cole and Elliss [10].

The acceleration of proteolysis in our experiments after 12 hours following the irradiation may be connected with the presence in the bone marrow at this period of a considerable amount of dead cells and cell detritus, the high order of accessibility of which to the action of proteolytic enzymes is known from the old work of Northrop [11].

The second rise of sensitivity of the proteins to the action of proteolytic enzymes may be noted after 6 and 13 days following the irradiation, when the death of cells is rather infrequent and the cell detritus as a rule is no longer detected in the bone marrow. We assume that this rise of enzymic cleavability of the proteins is connected with the synthesis of lower-polymeric proteins, with higher sensitivity to the action of the proteolytic enzymes.

It is rather probable that the death of cells in the first hours after irradiation and their high lability in the period of the initial regeneration are partly determined by the phenomenon discovered by us.

SUMMARY

Irradiation of rabbits with a 1000 r dose causes a rise of sensitivity of bone marrow proteins to the action of pepsin and trypsin.

The increase of enzymic cleavability of the bone marrow proteins, noticeable beginning with the first minutes after irradiation, rises somewhat during the next 12 hours. A second rise of sensitivity of bone-marrow proteins to the proteolytic enzymes was noted following 6 and 13 days after the action of ionizing radiation.

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A STUDY OF THIOCHROME-S^{35} METABOLISM IN THE ORGANISM OF WHITE RATS

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The study of the decomposition processes of thiamine in animal and human organisms is necessary for the understanding of the causes of origin of endogenous B_1-hypovitaminoses and for the development of dependable methods of determination of the lack of this vitamin in the human organism.

One of the paths of decomposition of thiamine is its oxidation in animal tissues to thiochrome. Titaev [1–3] has shown that the oxidation of thiamine into thiochrome is connected with the reduction of dehydroadrenaline and that it is catalyzed by the enzyme thiamine-dehydrogenase. Despite the fact that the enzymic nature of this process is in dispute [4], the possibility of oxidation of thiamine to thiochrome in the animal organism is beyond any doubt, especially after the studies in which radioactive thiochrome had been isolated, after the administration of labelled thiamine to rats, from urine [5] and the internal organs [6]. This compound does not have a vitamin activity [7, 8] and is a product of irreversible decomposition of thiamine.

Data from a study of the thiochrome-S^{35} metabolism and the products of its decomposition in the organism of white rats are presented in this paper.

METHOD

Thiochrome-S^{35} was prepared from Thiamine-S^{35} by oxidation of the latter with alkaline ferricyanide. In order to obtain pure specimens of thiochrome-S^{35}, the alcohol-soluble form of thiochrome-S^{35} was converted three times into S ^{35}-thiochrome-HCl by means of N HCl, this being water soluble [9, 10]. The purity of the resulting thiochrome-S^{35} was verified by ascending chromatography on paper using the solvent system of: isobutyl alcohol, methanol, formic acid and water (110:80:15:5), which had been suggested by Shnol' [11]. For this purpose 0.01 ml of a solution of S^{35}-thiochrome-HCl, in the amount of 30-50μg was deposited onto strips of chromatographic paper (2 × 55 cm) at the distance of 6 cm from the lower end of the strip. The paper strips were immersed into the partition solvent system (level – 0.5 cm) in closed cylinders and were left in a darkened chamber for 24 hours at room temperature. After a 10-minute drying, at 70° the chromatogram was divided into 110 equal portions (0.5 × 2 cm) by making needle prick marks on it. Then, the radioactivity of each area was counted on the strip by means of an end-window counter provided with an aluminum window with a 0.5 × 2 cm opening. After the counting of radioactivity, the chromatogram was examined by means of filtered ultraviolet light for identification of thiochrome-S^{35}. The results of one of the experiments are shown in Fig. 1.

In checking the purity of thiochrome-S^{35} repeatedly, we discovered, by radiochromatography, the presence in the specimen obtained by us of a second unidentified component (R 0.96) which comprised 4–6% of total S^{35} detected in the chromatograms, in addition to thiochrome-S^{35} (R 0.55) which corresponded to 94–96% of S^{35}.

Pure thiochrome-S^{35} was eluted from the appropriate regions of the chromatograms by means of 0.0001 N HCl. In order to obtain eluates with a sufficient concentration of thiochrome-S^{35}, the elution was run simultaneously with 30–40 rhombic sections cut from simultaneously prepared chromatograms and threaded onto a thread. The 0.0001 N HCl was delivered with a micropipette, dropwise, to the upper end of the thread. The thiochrome-S^{35} content was estimated by radioactivity and this was introduced subcutaneously into the rats, at dosage of 100 μg per animal.

The experiment was run on 40 male rats, weighing 200–225 g each, on a usual diet. The rats, after administration of thiochrome-S^{35}, received 50 ml whole milk daily per animal.

The excretion of thiochrome-S^{35} was determined in the urine of the experimental animals along with the other fractions of radioactive sulfur [13]; only total S^{35} was determined, after mineralization [12], in the feces and the internal organs.

For collection of urine and feces, the rats were placed in large porcelain Buchner funnels. It was established by special experiments that the thiochrome-S^{35} is stable, in pure solutions and in urine, after having been acidified with hydrochloric acid to pH 2–3 and kept for 48–72 hours at room temperature. Therefore, 2 ml of 0.1 N hydrochloric acid was added to each flask used for collection of the urine, in order to stabilize thiochrome-S^{35}.

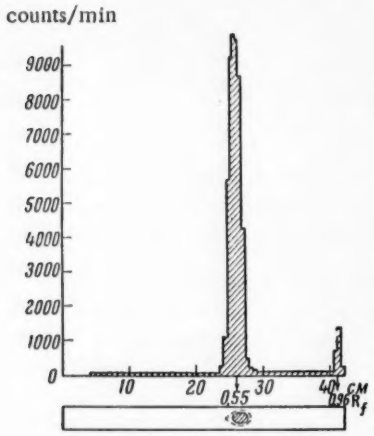

Fig. 1. Chromatographic and isotopic indication of purity of the prepared thiochrome-S^{35}.

Total S^{35} was determined in the urine by determination of radioactivity of 0.2 ml of urine which had been evaporated on a steel target at 80°. For the determination of thiochrome-S^{35}, the urine was centrifuged in Plexiglas test-tubes at 5000 rpm for 10 minutes. Then, 10 ml of the urine (pH 2–3) was extracted for 3 minutes with an equal volume of isobutyl alcohol, saturated with water, for the removal of alcohol-soluble radioactive and fluorescing impurities. During this, less than 1.0% of S^{35} present in the acidified urine passes into the isobutyl alcohol. After the removal of the alcohol, 2 ml of 30% sodium hydroxide solution, and 12 ml of isobutyl alcohol were added to the urine residue and the whole was energetically shaken for 2 minutes in a separatory funnel. After the separation of the alcohol and the alkaline residue, the latter was extracted once again with 8 ml of isobutyl alcohol. Both alcoholic extracts were combined and, after settling for a day, 2 ml of the transparent solution of thiochrome-S^{35} in isobutyl alcohol was evaporated on a steel target at 80°, with four depositions of 0.5 ml each of the alcoholic solution onto the target. In addition, a chromatographic examination of the urine was run.

The rate of excretion of total S^{35} in the urine of rats was studied in the preliminary experiments covering various time intervals after the administration of thiochrome-S^{35}. The results of these experiments are given in Table 1.

TABLE 1

Elimination of Total S^{35} in Urine After Administration of 100 μg of Thiochrome-S^{35} Each to White Rats. *

Interval after administration of thiochrome-S^{35}, in hours	First Rat		Second Rat	
	Radioactivity of total S^{35}			
	In counts/min	In % of administered dose	In counts/min	In % of administered dose
7	794 680	64.5	714 630	58.1
12	40 844	3.2	61 500	5.0
24	18 450	1.5	15 990	1.3
48	5650	0.4	4674	0.3
72	2412	0.4	4182	0.3

* Total radioactivity of the administered thiochrome was equal to 1,230,000 counts/min.

It is possible to say on the basis of these experiments, that S^{35} of thiochrome-S^{35} is rapidly eliminated with the urine after a parenteral administration. Here, the main bulk of S^{35} is excreted within the first 7 hours. The elimination of S^{35} in the urine during the subsequent hours is sharply curtailed and remains at a low level for the second and third days after the injection. Considering such data, we considered it possible to limit our selves in the following experiments to the study of the urine collected from the rats over 24 hours, following the injection of thiochrome-S^{35}.

Distribution of Radioactive Sulfur of Thiochrome-S^{35} In Organs and Tissues of White Rats. 24 hours following the injection of 100 μg of thiochrome-S^{35}, the animals were decapitated, the samples of organs and tissues were homogenized and mineralized to $BaSO_4 \cdot BaS^{35}O_4$, and weighed for calculation of self-absorption of the β-radiation from S^{35} [14]. In order to estimate the intensity of the absorption of thiochrome-S^{35} by the organs and the tissues, we made calculations of the relative specific activity (RSA) [15] by the formula:

$$RSA = \frac{\text{counts/ min in 1 g of tissue} \cdot 100}{\text{counts/ min, introduced per 1 g of animal weight}}$$

TABLE 2

RSA of Tissues Following 24 Hours After Injection of 100 μg of Thiochrome-S^{35} to Each Rat (average data for 16 rats)

Suprarenal	Kidneys	Skeletal muscle	Liver	Heart	Brain	Small intestine
16.8 * (9.1–23.0)	14.7 (9.0–16.5)	7.3 (5.2–9.4)	4.8 (4.0–5.2)	4.0 (3.3–5.0)	3.9 (3.0–4.7)	2.1 (1.6–3.1)

* The limits of variations are shown in parentheses

The results, shown in Table 2, indicated that the S^{35} of the subcutaneously administered thiochrome-S^{35} is retained only to an insignificant degree in the internal organs and tissues of the rats. In the determination of only the total S^{35} in the tissues, we cannot relate all of its radioactivity to the thiochrome. A comparison of the RSA of the various organs and tissues revealed some considerable differences in the intensity of retention of thiochrome sulfur in the various tissues. Relatively more S^{35} is detected in the suprarenals and the kidneys. It is possible to suggest a retention of part of S^{35}, which is eliminated with the urine, in the last instance.

Elimination of Thiochrome-S^{35} and its Sulfur-Containing Metabolites With the Urine and the Feces. We concentrated our main attention, during the study of thiochrome metabolism, on the examination of urine, since the predominant part of S^{35} of the thiochrome-S^{35} administered to the rats is eliminated with the urine. Each experiment was set up with eight rats, collecting their urine for 24 hours, and the total urinary S^{35}, thiochrome-S^{35} and sulfate radioactivity were determined. The results of these experiments are given in Table 3.

The results thus obtained indicate that over 24 hours after the administration of 100 g of thiochrome-S^{35} to each of the white rats, on the average 70.7% of S^{35} is eliminated with the urine. Such a high percentage of elimination of total S^{35} with the urine indicates a more rapid excretion of thiochrome from the organism in comparison with thiamine-S^{35} [16, 17]. Only 31.8% of unchanged thiamine-S^{35} is eliminated on the average with the urine over 24 hours. This fact indicates a decomposition of the thiochrome in the rat organism and the excretion of its sulfur-containing metabolites with the urine. Radioactive sulfate was detected in the urine as the terminal product of oxidation of the sulfur of thiochrome-S^{35}. On the average, 1.5% of radioactive sulfates, relative to the entire amount of administered thiochrome-S^{35}, was eliminated over 24 hours, which amount is considerably smaller than that found after administration of thiamine-S^{35} to the rats [18]. This comparison indicates a slower decomposition of thiochrome in the organism and a slower oxidation of its sulfur to the terminal products, as compared with thiamine.

The intensity of decomposition of thiochrome-S^{35} in rat organism and the excretion of its metabolites in the urine, is more clearly shown after a study of the percentage proportion of the fractions of urinary S^{35} studied

by us. For the calculations we took as 100% the total S^{35} of the urine and by subtracting from this the urinary thiochrome-S^{35} and the urinary radioactive sulfates, we found the percentage content of "neutral" S^{35} formed in the decomposition of thiochrome-S^{35}.

TABLE 3

Urinary Excretion of Thiochrome and Its Sulfur-Containing Metabolites After Administration of 100 μg of Thiochrome-S^{35} to Each Rat

Counts/min of administered thiochrome-S^{35}	Eliminated in urine over 24 hours					
	In counts/min			In % based on administered dose		
	Total S^{35}	Thiochrome-S^{35}	$S^{35}O_4$	Total-S^{35}	Thiochrome-S^{35}	$S^{35}O_4$
1 138 000	785 000	386 920	7966	69,0	34,0	0,7
»	699 870	281 080	13 656	61,5	24,7	1,2
»	685 076	231 010	28 450	60,2	20,3	2,5
»	912 360	569 000	14 794	80,7	50,0	1,3
»	772 700	415 370	25 036	67,9	36,0	2,2
»	864 880	372 120	15 930	76,0	32,8	1,4
»	787 496	520 066	15 930	69,2	45,8	1,4
»	632 728	175 010	26 174	55,6	14,5	2,3
900 600	565 570	117 980	13 509	62,8	13,1	1,5
»	693 460	180 120	25 216	77,0	20,0	2,8
»	729 480	330 426	27 918	81,0	37,6	1,3
»	453 900	138 690	13 500	50,4	15,4	1,5
»	576 380	421 480	19 810	64,0	48,6	2,2
»	614 200	298 100	—	68,2	33,1	—
»	562 875	230 550	18 012	62,5	25,6	2,0
»	552 060	228 650	13 500	61,3	25,4	1,5
615 000	507 990	300 730	5535	82,6	48,9	0,9
»	410 820	217 710	11 680	66,8	35,4	1,9
»	465 550	194 850	—	75,7	31,7	—
»	418 815	274 804	7995	68,1	44,7	1,3
»	348 705	128 690	11 070	56,7	20,6	1,8
»	392 985	142 060	15 374	63,9	23,1	2,5
»	467 010	189 420	7 380	76,1	30,8	1,2
»	516 600	237 000	7 996	84,0	38,7	1,3
398 000	240 390	101 490	5 572	60,4	25,5	1,4
»	262 280	189 050	2 786	65,9	47,5	0,7
»	266 660	127 360	3 980	67,1	32,0	1,0
»	253 128	106 266	7 164	63,6	26,7	1,8
»	248 350	105 470	5 970	62,4	26,5	1,8
»	336 810	190 244	3 184	84,5	47,8	0,8
»	261 480	136 510	4 756	65,7	34,3	1,2
»	269 910	130 540	5 572	74,6	32,8	1,4
2 544 000	1 704 180	1 109 180	33 072	67,0	43,6	1,3
»	1 470 400	552 050	48 836	57,8	21,7	1,9
»	1 653 600	615 650	81 410	65,0	24,1	2,3
»	1 802 250	755 570	25 442	74,0	29,7	1,0
»	2 124 200	1 012 510	25 442	83,5	39,8	—
»	1 568 560	676 700	40 704	61,5	26,6	1,6
Average				70,7	31,8	1,5

After the calculations based on the results of 38 experiments, we obtained the following data:

S^{35} fractions	Radioactivity in %
Total S^{35}	100
Thiochrome-S^{35}	44.9
"Neutral" S^{35}	53.0
Sulfate S^{35}	21

Thus, the results of examination of the urine indicated that about half of the administered thiochrome-S^{35} is decomposed in the organism of white rat and its metabolites, containing S^{35}, are eliminated with the urine.

For the clarification of the amount and the nature of the intermediate decomposition products of thiochrome, we made use of chromatographic analysis of the urine. For this purpose we administered thiochrome-S^{35} with a high specific activity (Table 3) to six rats in the last experiment.

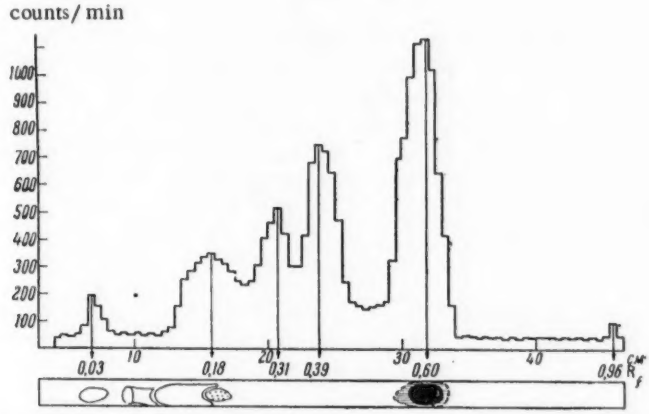

Fig. 2. The radiochromatogram of 0.1 ml of urine, collected over 6 hours after administration of 100 g of thiochrome-S^{35} with total radioactivity of 2,544,000 counts/min to the rats.

In order to insure high concentrations of the radioactive products, which would be sufficient for counting the radioactivity of the chromatogram, we used for the analysis a portion of urine that was collected over the first 6–7 hours following the injection of thiochrome-S^{35}, during which period the greater part of administered S^{35} is eliminated (Table 1). For the study proper, 0.1 ml of a transparent urine (after 10 minutes of centrifuging at 5000 rpm) was deposited in drops onto a strip of chromatographic paper by means of a micropipette. The evaporation of the urine was accelerated by blowing of the area of deposition with a stream of dry nitrogen preheated to 70–80°. The chromatographic analysis was run by the above-given technique with calculation of the radioactivity along the chromatogram. In all, 12 chromatograms were made, two for the urine of each rat.

The radiochromatographic analysis gave well-reproducible results and six radioactive components were detected on all chromatograms. The results of one of the experiments are shown in Fig. 2. Two of the six radioactive components, detected on the chromatograms, were identified by means of radioactive indicators – thiochrome-S^{35} (R_f 0.60) and radioactive sulfate (R_f 0.03). Thiochrome was identified also by its bright-blue fluorescence in the spot on the chromatogram under filtered ultraviolet light. One of the components (R_f 0.18) had a weak-blue fluorescence in the undeveloped chromatogram, this fluorescence being covered by the green-yellow fluorescence of the other impurities in the urine.

Three components (with R_f 0.31, 0.39 and 0.96) did not fluoresce in ultraviolet light either before or after oxidation of the chromatogram with an alcoholic alkaline ferricyanide solution. In considering the composition of the partitional mixture, it is possible to note that the metabolites of thiochrome-S^{35} have different solubilities in alcohols, these metabolites having been found in the urine. Among them there are components which are very readily soluble in isobutyl alcohol and methanol (R_f 0.96), then thiochrome-S^{35} follows, and the components with R_f 0.39, 0.31 and 0.18, are last.

It is evident during an examination of the quantitative relationship of the sulfur-containing metabolites of thiochrome-S^{35} on the above-mentioned chromatogram, that thiochrome-S^{35} greatly predominates over the other components, by comprising 38.5% of the total S^{35}. Then, in descending order, follow the components with R_f 0.39 (24.1%), 0.31 (20.0%), and 0.18 (16%). The radioactive sulfate and the alcohol-soluble component with R_f 0.96 make up a very small percentage of the total radioactivity of the chromatograms (1.1 and 0.25%, respectively). It should be stressed that the radiochromatographically detected relationships between thiochrome and radioactive sulfates agrees with the data obtained in the determination of sulfates by $BaS^{35}O_4$, and that of thiochrome-S^{35} by

extraction with isobutyl alcohol. It is possible to suppose, in consideration of this correlation, that the components with R_f 0.18, 0.31 and 0.39 are compounds comprising the "neutral" S^{35} of the urine.

We also determined the elimination of S^{35} with the feces for some of the test rats over 24 hours following the administration of thiochrome-S^{35} to them.

For this purpose, the content of total S^{35} was determined in the feces collected from the bottom of the Buchner funnel as well as in the contents of the large intestines.

The excrements under study were weighed, homogenized and mineralized for the determination of total S^{35}. The determination of radioactivity was done by the already-described technique and the percentage of elimination of S^{35} with the feces was calculated relative to the administered dose of thiochrome-S^{35}. The results obtained from four rats showed that over 24 hours there is eliminated with the feces from 3.3 to 7.0% (4.9% on the average) of S^{35} while from 3.8 to 10.5% (6.2% on the average) remains in the contents of the large intestine, based on the radioactivity administered to the animal. In all, on the average 11.1% of S^{35} is eliminated over 24 hours with the excrement.

SUMMARY

Thiochrome-S^{35}, administered parenterally to white rats, is rapidly eliminated with urine and feces. 24 hours following the administration of 100 g of thiochrome-S^{35} 8.8% of S^{35} is found on the average in the internal organs and tissues, 70.1% in the urine and 11.1% in the excrement.

S^{35} is distributed nonuniformly among the internal organs of white rats; most of it is found in the suprarenals and the kidneys.

Fractionation of the radioactive sulfur-containing compounds of the urine showed that the unchanged thiochrome-S^{35} makes up on the average 44.9% of these substances, "neutral" S^{35} – 53.0%, and radioactive sulfate – 2.1%.

Four more radioactive metabolites of thiochrome-S^{35} were detected in the urine, in addition to thiochrome-S^{35} and radioactive sulfate, by means of radiochromatographic analysis.

It is possible to consider, on the basis of this work, that thiochrome is but an intermediate product of thiamine metabolism and, in turn, decomposes in the rat organism with the terminal formation of sulfates.

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BIOGENESIS OF NICOTINE AND TRANSFORMATION OF NITROGENOUS SUBSTANCES IN TOBACCO SPROUTS

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The biogenesis of nicotine and of the alkaloids which accompany it is rather incompletely understood to this day; the process of formation of alkaloids in living organisms, in general, has not been studied sufficiently.

The concept concerning the possible participation of some amino acids in the synthesis of nicotine, as well as the fact of formation of the latter from the decomposition products of protein (along with experiments run under conditions which were close to those of a living cell) served as a useful starting point for setting up a series of subsequent studies in this direction [1–4].

Thus, the application of the method of tagged atoms showed that nicotine, labelled at the carbon atom, may be readily obtained by growing tobacco plants in an atmosphere that contains radioactive carbon dioxide as well as in aqueous cultures, into the nutrient mixture of which, carbonate with labelled carbon or some other organic compounds had been introduced appropriately [5].

Byerrum and co-workers[6, 7] isolated radioactive nicotine through the entry of labelled carbon into the N-methyl group of nicotine by growing Nicotiana rustica plants in a nutrient mixture containing carbon-labeled radioactive compounds – methionine, choline, glycine, formic acid. It appeared that the most active source for the formation of the methyl group of nicotine are methionine and choline; glycine and formic acid are incorporated into the nicotine molecule considerably more weakly.

Dewey et al [8] also showed that the methyl group of methionine is transferred into the N-pyrrolidine ring of nicotine in the process of transmethylation.

Dewey et al [9] and Leete [10] isolated radioactive nicotine after the administration of ornithine-2-C^{14} with the nutrient mixture to tobacco plants and in this way proved that ornithine is a precursor of the pyrrolidine ring of nicotine.

Leete [11] introduced lysine-2-C^{14} into the nutrient mixture in which Nicotiana tabocum and Nicotian glauca was being grown and showed that this substance is not a source of the pyridine ring of nicotine and anabasine. The entire radioactivity of anabasine was concentrated at the α-carbon atom of the piperidine ring of anabasine.

Bothner-By et al [12] obtained nicotine with a small content of N^{15} or C^{14} in the pyrrolidine ring, by running experiments with sterile cultures of roots of tobacco and with introduction of L-(+)-lysine-N^{15} or L-(+)-lysine-C^{14}, labelled in all positions, and established that the formation of the pyridine ring does not occur from lysine and that lysine does not lie in the direct path of nicotine biosynthesis.

Dawson et al [13] treated sterile cultures of isolated tobacco roots by specimens of nicotinic acid, labelled with tritium in the ring, and by specimens of nicotinic acid, labelled both in the ring and in the carboxyl group with C^{14}. The resulting nicotine was radioactive; it was supposed that the pyridine ring of nicotinic acid is a precursor of the pyridine ring of nicotine, nornicotine and other tobacco alkaloids.

Byerrum and co-workers[14, 15] also showed that the α-carbon atom of glycolic acid is incorporated into the N-methyl group of nicotine; formaldehyde and the β-carbon atom of serine are incorporated into the N-methyl group of nicotine in the process of metabolism in the tobacco plant.

TABLE 1

Amino Acid Content in Proteins of Tobacco Sprouts (In %)

Protein fraction	Albumin				Globulin				Glutein			
Names of amino acids	Duration of sprouting in days											
	0	3	5	10	0	3	5	10	0	3	5	10
Aspartic acid	7,5	7,9	9,2	9,5	9,8	8,3	5,5	7,7	7,4	8 1	8,1	6,9
Glutamic acid	20,3	19,8	10,0	14,6	28,0	26,8	20,0	17,5	22,0	21,8	14,2	15,0
Serine	3,3	3,5	3,9	3,3	4,0	4,5	3,5	7,0	4,3	4,0	4,0	3,7
Glycine	4,6	4,3	4,0	3,0	5,5	4,3	3,4	5,6	4,0	5,7	5,7	3,4
Threonine	2,5	3,0	4,2	3,9	4,8	4,6	3,5	3,6	5,5	4,1	5,6	3,6
Alanine	3,5	4,0	6,5	8,1	4,3	4,4	4,5	8,1	3,5	5,0	7,3	3,6
Valine	3,6	4,0	4,3	8,3	3,9	4,0	4,3	7,5	3,7	7,0	7,3	4,4
Tyrosine	1,3	1,4	1,4	3,3	0,7	0,8	0,6	1,7	1,4	2,0	2,9	1,9
Methionine	0,9	1,0	1,3	1,8	0,7	0,7	0,7	0,4	1,0	1,3	1,6	1,1
Leucine	7,2	7,0	9,1	11,5	8,5	8,5	8,21	13,2	7,3	13,9	14,0	14,7
Phenylalanine	4,4	5,0	7,0	12,7	4,4	4,4	4,4	5,9	4,2	6,4	7,1	5,3
Arginine	9,2	9,3	10,1	9,6	10,0	7,9	8,3	7,8	10,1	10,9	8,8	9,7
Histidine	1,9	2,3	2,2	2,6	2,6	2,3	2,4	3,1	2,4	3,4	2,9	3,0
Lysine	5,2	4,5	4,6	4,0	3,3	2,6	2,4	2,2	4,4	6,5	4,1	4,4
Proline	2,2	2,7	3,4	3,4	2,2	2,7	2,8	3,0	5,9	5,8	5,2	5,2
Cystine	1,9	2,1	2,0	2,1	3,0	1,9	1,7	1,5	3,4	3,6	3,2	3,1
Tryptophan	1,8	1,4	2,4	1,6	1,5	1,2	1,1	0,9	1,4	1,3	1,5	1,4

TABLE 2

Amount of Free Amino Acids In The Sprouts
In mg % of Total Nitrogen

Names of amino acids	Duration of sprouting in days				Names of amino acids	Duration of sprouting in days			
	0	3	5	10		0	3	5	10
Aspartic acid	2,6	6,7	22,2	0,6	Valine	11,3	58,5	73,8	55,7
Glutamic acid	47,1	37,0	30,6	3,8	Methionine	2,7	11,6	24,6	24,0
Serine	2,1	10,3	118,7	11,8	Leucine	8,2	83,0	119,7	62,6
Glycine	8,8	8 8	143,1	55,7	Phenylalanine	4,1	18,6	46,0	22,6
Threonine	7,9	27,8	27,4	19,5	Arginine	5,3	6,6	12,2	18,4
Alanine	15,3	123,0	214,8	192,5	Lysine	7,5	6,8	29,7	29,7
Tyrosine	11,2	107,0	337,1	280,9	Histidine	2,5	3,0	19,0	9,3

The papers cited above are the signposts on the road to the close study of the problem of alkaloid biosynthesis and, especially, for the setting up of the problem about the connection and interrelation of the process of formation of nicotine with the dynamics and transformation of nitrogenous substances. Specifically, such a interrelationship was established by us in the study of the processes of sprouting and ripening of tobacco seeds, and it was shown that during this process the synthesis and the decomposition of nicotine proceed in close correspondence with the transformation of the protein substances and amino acids [16].

For the purposes of a further study of this process, we ran some experiments with the use of paper chromatographic method for the determination of the content of bound and free amino acids at various stages of sprouting of tobacco seeds in order to clarify and to establish the possible role of some of them in nicotine biosynthesis.

EXPERIMENTAL

We ran the seed sprouting in light in moist chambers at room temperature. The sprouts were kept briefly at the temperature of 70° for the inactivation of enzymic activity, after which the seeds were dried at the temperature of 30–40° to air-dry state. The material was macerated in a coffee mill and was extracted with ethyl ether in a Soxhlet apparatus for 8 hours for the removal of fat.

Isolation of Proteins

The extraction of proteins was done by successive treatment with various solvents.

The extraction of albumin was done by a tenfold volume of distilled water for three hours with constant stirring. The extraction was run three times at the temperature of 5–8°. Toluene was used as the antiseptic. The resulting extract was filtered off. The albumin was precipitated with alcohol used in 1:2 proportion. Globulin was isolated from the same sample after the removal of the albumin.

Globulin was extracted with 5% sodium chloride solution and was precipitated by electrodialysis. Platinum plates served as the electrodes; current strength was 25 ma; potential used was 18 V; duration of the electrodialysis was 5 hours. Globulin was isolated in the crystalline state.

For the extraction of glutein, the sample was treated with 0.5% sodium hydroxide solution, filtered and the protein was precipitated from the resulting filtrate by acidification with dilute acetic acid to pH 4.6.

The precipitates obtained from three protein fractions were filtered off, washed and dried lyophilically. The specimens were stored in a desiccator over phosphoric anhydride.

The free amino acids were extracted by extraction of separate samples of the original seeds and tobacco sprouts with 70% ethanol over 100 hours with constant stirring. Ethanol and water were distilled off under vacuum. The dried amino acids were dissolved in ml of distilled water and were kept at 0° with the antiseptic.

Samples of the protein, containing approximately 1.6 mg of nitrogen, were hydrolyzed by being boiled for 24 hours with 1 ml of 6N hydrochloric acid. The insoluble humic substances were filtered off after the end of hydrolysis and were washed with 2 ml of hot distilled water. The wash waters were added to the hydrolyzate. Excess hydrochloric acid was removed in a vacuum desiccator over KOH and P_2O_5 after many additions of water. The hydrolyzate was adjusted to pH 6.0, evaporated to dryness and taken up in 1 ml of distilled water. The resulting hydrolyzate was stored at 0° with a drop of toluene.

Alkaline hydrolysis was run for the determination of tryptophan: about 20 mg of the protein was hydrolyzed by 1 ml of 20% sodium hydroxide solution for one hour. The determination was performed by the generally accepted method [17].

The method of two-dimensional paper chromatography [18] was used for the identification of the amino acids and for the estimation of their approximate amounts in the experimental samples. We used the "Leningrad slow-filtering" paper, with dimensions of 40 × 40 cm. The paper was washed free of metal ions by 3% hydroxyquinoline solution in n-butyl alcohol-acetic acid mixture. For the solvent we used the system: n-butyl alcohol-acetic acid-water (4:1:1) and phenol saturated with water.

Quantitative Chromatography

The method of descending paper chromatography was used for the quantitative determination of amino acids. Mixtures of various solvents were used for the separation of the amino acids: 1) phenol, distilled, saturated with phosphate buffer (pH 12; 0.067 M); here we used paper saturated with the same buffer; this solvent gave (according to MacFarren [19])a good separation of aspartic and glutamic acids, serine and glycine. 2) o-Cresol, freshly distilled, saturated with phosphate buffer (pH 6.2; 0.067 M); the paper was treated with the pH 6.2 buffer; with this solvent we determined alanine, tyrosine, valine, methionine, leucine and phenylalanine [19]. 3) A mixture of butyl alcohol–acetic acid – water (4:1:1) served for the determination of cystine, lysine, arginine, proline and histidine.

In all cases, the test mixture of amino acids was deposited on the chromatographic paper in the form of a stripe, as recommended for a better separation [20]. In this case, the cystine was determined directly after hydrolysis in order to avoid any degradation. The separation of lysine was attained after a fivefold passage of the solvent.

The time of drying of the chromatograms from traces of solvents was strictly constant. Phenol and cresol were removed at 60° over 1.5 hours in a steady air stream. 2% ninhydrin solution in acetone was used for the developer, the acetone containing 1% of acetic acid. The paper was pulled through the ninhydrin solution in the direction opposite to that of solvent motion. For the development of amino acid colors, the chromatograms were heated at 60° for 30 minutes. The amino acid spots, colored by ninhydrin, were cut out and eluted. A 0.2% cadmium chloride solution in 40% methanol [21] was used for the elution of the developed amino acids and

for simultaneous fixation of the color. Time of elution – two hours with constant stirring. The optical density of the color of the solution was determined by an SF-4 spectrophotometer at $\lambda = 510$ mμ. The amount of the amino acids in the experimental samples was determined by comparison with standard mixtures. The concentration of the standard mixtures were approximately adjusted to the concentration of the experimental samples [22]. In each case the experimental and the standard mixtures were deposited alternately on the same sheet of paper. A number of other specific developers were used as developers along with ninhydrin.

TABLE 3

Nicotine content in the Sprouts (In %)

Duration of sprouting, in days	Nicotine, in %	M.p. of nicotine dipicrate
0	0	–
3	0	–
5	0.22	217
10	0.67	218

Proline was identified by its specific reaction with isatin. The 0.4% isatin solution in n-butyl alcohol, containing 0.4% of acetic acid [23] was used as the developer For development of color of the chromatogram, the latter was heated to 105° for 15 minutes. Here, proline gave a spot with an intensely blue color on a bright yellow background. The intensity of color of the spots was measured directly on the paper with Shipalov-model densitometer. A calibration curve was constructed for the quantitative determination of the content of proline in the experimental samples.

We used the method of Rosenberg, Ennor and Marrison [24] for the determination of arginine, according to which method arginine forms a red-violet complex in an alkaline solution with diacetyl and 1-naphthol. The optical density of the color of the solution was measured at $\lambda = 535$ mμ. Histidine was determined by the method of Fraenkel-Conrat and Singer [25] (reaction with diazotized sulfanilic acid).

RESULTS OF THE INVESTIGATION

Fifteen amino acids (see figure) were identified by means of the two-dimensional chromatography in the hydrolyzates of the proteins.

The data on the quantitative content of bound amino acids in the proteins are given in Table 1.

The analytical data given in Table 1 indicate that a number of amino acids (serine, glycine, methionine, histidine and proline) do not suffer any noticeable changes during the sprouting period. The content of the other amino acids (alanine, valine, phenylalanine and leucine) rises considerably during the sprouting period, especially at the 10th day, but does so irregularly. The amount of glutamic acid, in all protein fractions, and that of arginine, only in the globulin fraction, are decreased noticeably.

It was interesting to clarify the dynamics of the changes in the content of free amino acids in this process, in connection with the data obtained concerning the content of amino acids in the proteins at various stages of sprouting of the tobacco seeds (Table 2).

The resulting data indicate that the content of free amino acids (except for glutamic acid) increases considerably during the sprouting process, reaching a maximum in the five-day sprouts. This increase is caused by the active hydrolytic processes. The content of free amino acids in the ten-day sprouts is somewhat decreased, which fact is evidently connected with the predominance of synthetic processes in them.

Glutamic acid occupies the key position in nitrogen metabolism and, as an active metabolite, this acid differs sharply in its mobility from the other amino acids.

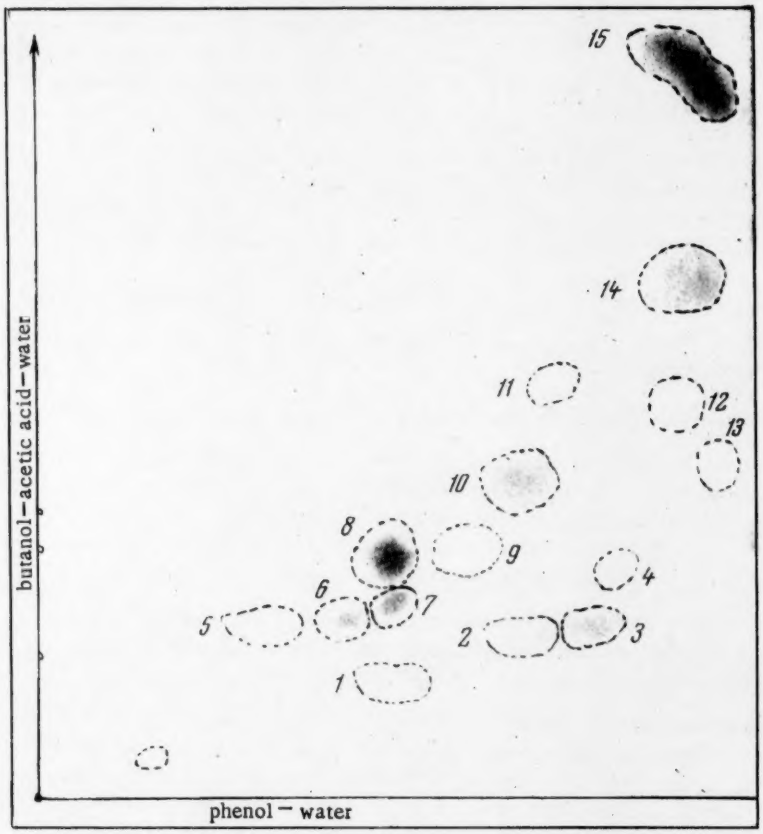
The glutamic acid content, in contrast to the other amino acids, is reduced considerably in the process of sprouting of the tobacco seeds.

Nicotine was determined as the dipicrate [26]. The results shown in Table 3 indicate that nicotine is absent from the dormant ripe tobacco seeds, but is formed during the swelling and the sprouting of tobacco seeds when the cleavage of proteins occurs, under the influence of entry of water and the subsequent action of proteolytic enzymes, with formation of free amino acids and their further transformation in the process of embryonic development.

Evidently, the origination of nicotine occurs in the process of protein synthesis in the growing tissues and is localized in the rootlets as shown by Schmid and Serrano [27] for tobacco sprouts in their transition from the heterotropic to autotropic nutrition.

It is evident, as a result of our experiments, that a definite connection exists between the processes of nicotine biosynthesis and the content of glutamic acid in the sprouts; specifically, the amount of bound and free glutamic acid decreases noticeably, while the nicotine content increases, during the sprouting of tobacco seeds. The glutamic acid, liberated as a result of hydrolytic processes, is not accumulated in the sprouts and its quantity decreases. The decrease of the amount of bound and free amino acid may be also caused by its participation in the synthesis of alkaloids. The predominance of glutamic acid in the proteins of the seeds and its subsequent decrease in the sprouts is evidently connected with its transformation into other compounds, specifically into alkaloids, in part.

For the final solution of this problem, the setting up of further studies is needed, especially those with the use of labelled glutamic acid which would permit one to give an answer to this question.*



Two-phase chromatogram of the hydrolyzate of tobacco-sprout globulin. 1) Cystine; 2) histidine; 3) lysine; 4) arginine; 5) aspartic acid; 6) serine; 7) glycine; 8) glutamic acid; 9) threonine; 10) alanine; 11) tyrosine; 12) tryptophan (trace); 13) proline; 14) phenylalanine; 15) leucine.

* After the manuscript of this paper had been submitted to the press, a study was published in which the inclusion of D,L-glutamic acid-2-C^{14} was shown to take place in the pyrrolidine ring of nicotine [28].

SUMMARY

The study showed that the qualitative composition of amino acids in all protein fractions and during the entire duration of the seed sprouting period of tobacco remains constant; small changes in the content of alanine, valine, phenylalanine and leucine are observed in the direction of irregular rise; the amount of arginine (in the globulin fraction) is decreased noticeably; however, the amount of glutamic acid declines regularly in all protein fractions. The amount of the remaining amino acids, which enter the protein composition, remains constant.

The content of free amino acids, except for glutamic acid, increases regularly in the process of seed sprouting and reaches a maximum value in the five-day sprouts while the content of glutamic acid decreases constantly.

The decrease in the content of glutamic acid in the process of tobacco seed sprouting and the appearance and gradual accumulation of nicotine, which occurs in parallel with this, permit us to draw conclusions, as a result of which our comprehension is widened and the possibilities of further study of plant alkaloid biogenesis are clarified.

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CONCERNING SOME PECULIARITIES OF METABOLISM OF AN ANIMAL ORGANISM AFFLICTED WITH TUMOR

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In recent years there has appeared in the literature a number of papers in which experimental material is cited which proves the existence of some peculiarities in the metabolism of the tumor tissue. Thus, Laird and Barton [1], Butler and coauthors [2], Werkheiser and Visser [3], Schmitz and coauthors [4], Steckerl [6], Manoilov [7], Zil'ber and coauthors [8] and a number of other investigators have shown that the nucleoprotein metabolism is considerably different in the malignant tumors in comparison with the metabolism of the normal tissues.

There are also some data [9-13] on the changes in the protein metabolism.

A special interest exists in the paper by Warburg [14] and his followers [15-18] who regard that the beginning of the tumorous growth begins as a result of an irreversible affliction of the tissue respiration.

Along with these papers there are also some studies in which the metabolism was studied in an animal organism prior to the appearance of a tumor in it. Radringner and coauthors [19] and Täufel and coauthors [20] found a change in the nucleic metabolism and a lowering of citric acid synthesis in the liver prior to the development of a hepatoma. Mortreinil and coauthors [21] observed an increase of incorporation of P^{32} into nucleic acids of the nuclei of the animal liver, after the animal had been given a cancerogenic substance. Waravdekar and coauthors [22] showed a lowering of DPN in the liver of an animal receiving p-dimethylaminoazobenzene. Kühl [23] notes the change in the ratio of albumins to globulins in the serum, owing to the increased content of α- and β-globulins after smearing of the animal skin with cancerogenic hydrocarbons. Weiler [24] observed the disappearance of the antigen of the liver tissue with the development of a hepatoma. Cerecedo and others [10] have established that the content of RNA is increased and the amount of arginine and histidine is decreased, especially in the liver, with the development of a sarcoma in the lungs, liver and the spleen. Hollő and Zlatarov [25] discovered a drop in DNA by 40–50% in an animal organism following a skin smear to methylcholanthrene. Reiss and coauthors [26] note that an increased content of vitamins A, B_1 and C and a decline of the content of vitamin B_2 are observed on the fourth day following the administration of a cancerogenic hydrocarbon, these changes being observed in the liver.

Thus, we must regard as having been sufficiently cleared, by this time, the question about the occurrence of significant changes in the metabolism in animal tissues and organs after an entry of the cancerogenic substances into the animal organism and long before the development of a tumor.

Considerably fewer data are found in the literature about the effect of transplantation of a tumor on the metabolism of the animal organism.

In the present work we give some data on a study of some aspects of protein metabolism and nucleoprotein metabolism in the liver, spleen, kidneys and the brain at various intervals after the beginning of a transplant up to the appearance of the tumor. The protein metabolism was studied by means of carbon-labelled bicarbonate, while the nucleoprotein metabolism was studied by the rate of incorporation of inorganic radioactive phosphorus into the nucleic acids.

Hastings and co-authors [27], and later Wood [28], Ochoa et al [29] have clarified the paths of binding of carbon dioxide in an animal organism by the use of the labelled bicarbonate. The necessity of participation of carbon dioxide in many biochemical processes of the liver, spleen and other organs has become clear as a result of these studies. For this reason, more and more papers are appearing currently in the studies of the rate of in-

corporation of carbon dioxide into various organic compounds of the animal organism in normal and pathological states.

EXPERIMENTAL

The work was done with Crocker mouse sarcoma.* For the experiment we took unbred mice, weighing 18-20 g. Five to seven days prior to the tumor transplantation, the animals were placed into special cages in order to observe the amount of consumed food and the weight of excreted feces and urine. Over the entire experimental period, the animals were weighed daily. After the weight of a mouse had ceased to increase, half of the animals were transplanted with Crocker sarcoma. Usually the tumors formed following 10–14 days after the beginning of the transplant.

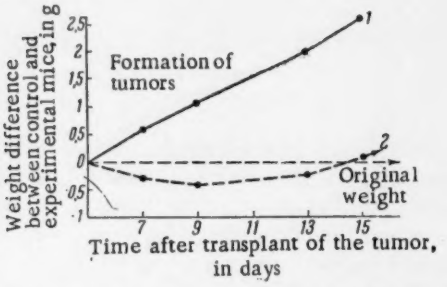

Fig. 1. The curve of mean weight difference between experimental (1) and control animals from the beginning of transplant of the tumor.

In all, 100 control and 100 experimental mice were used in the experiment. Changes in the weight of the animals after the transplantation of the tumor into them are shown in Fig. 1, from which it is evident that the experimental mice ate somewhat less food than did the controls, and correspondingly eliminated less urine and feces, but their weight was greater than that of the controls. On the average, the weight difference per mouse toward the end of the observation was about 2 g.

Naturally, the question arises: owing to what does the increase of the weight of the animal, in which the tumor is growing, occur?

To clear up this fact, we ran a weighing of individual organs in another series of experiments – taking livers, spleens and kidneys. It turned out that by the tenth day after the transplant the weight of the liver in the experimental animals was higher on the average than that of the controls by 14%, the weight of the spleen by 24% and the weight of the kidneys by 7%. This increase was caused by the large amount of solid residue. It was shown by the determination of total nitrogen in them, that the amount of it in the organs taken from experimental animals on the tenth day after the transplant, was greater than in the organs of the control animals. Thus, the solid residue of the liver of control animals contained 10.76% nitrogen, and 12.1% in experimental animals; in the spleen in control animals – 12.9%, in experimental animals – 14.36%; in the kidneys of control animals – 10.6%, in experimental animals – 11.67%. Each figure is the average of data obtained from 100 mice. Similar data are cited by Babson [30].

Consequently, the weight of individual organs increases during the growth of the tumor in an animal organism, owing to the increase of the protein mass in them.

An analysis of these facts led us to the supposition that a more intense incorporation of carbon dioxide into the protein substances of the organs and the tissues occurs in the course of tumor growth in an animal organism. To clarify this point we set up some experiments with bicarbonate with radioactive carbon.

The carbon-labelled bicarbonate was administered in the amount of 50 μ curies per animal, with a minimum amount of a carrier. Each time, along with the experimental animals, we made a study on the same number of control animals. Following 30, 60, 120, 180 and 240 minutes after the introduction of radioactive carbon, the animals were killed (for each interval, not fewer than five mice were taken). The organs were rapidly removed from them, and these were thoroughly macerated in the cold to a homogeneous mass. For the complete removal of acid-soluble fractions and free carbon dioxide, a manifold washing of the precipitate with acidified water was done. In order to be certain that no more carbon dioxide remained in the solid residue, the latter was placed, after having been washed, in a Warburg apparatus in which it was shaken for one hour in the presence of 10% potassium hydroxide solution. In not a single case did we determine any radioactivity in the alkaline solution. This indicates that all the free bicarbonate was extracted from the precipitate during the washing of the tissue. Then, in one series of experiments, the solid tissue residue was dried to constant weight; in another series, the proteins were precipitated by means of

* The Crocker sarcoma was obtained from the Laboratory of Oncological Institute, to the Director of which, Corresponding Member of the Academy of Medical Sciences, USSR, Professor L. M. Shabad, we express our great gratitude.

10% trichloroacetic acid. The resulting precipitate was dried to constant weight. In both cases, the total nitrogen was determined by the Kjeldahl method.

The specific activity per 25 mg of dry weight was calculated by means of the formula $(n_o - n_b)/n_b$, where n_o is the number of counts of the test material, and n_b the number of counts of the background. The counting of impulses was done with the end-window counter (1.5 cm diameter) on a counting set-up of Type B. The layer thickness was over 0.2 mm.

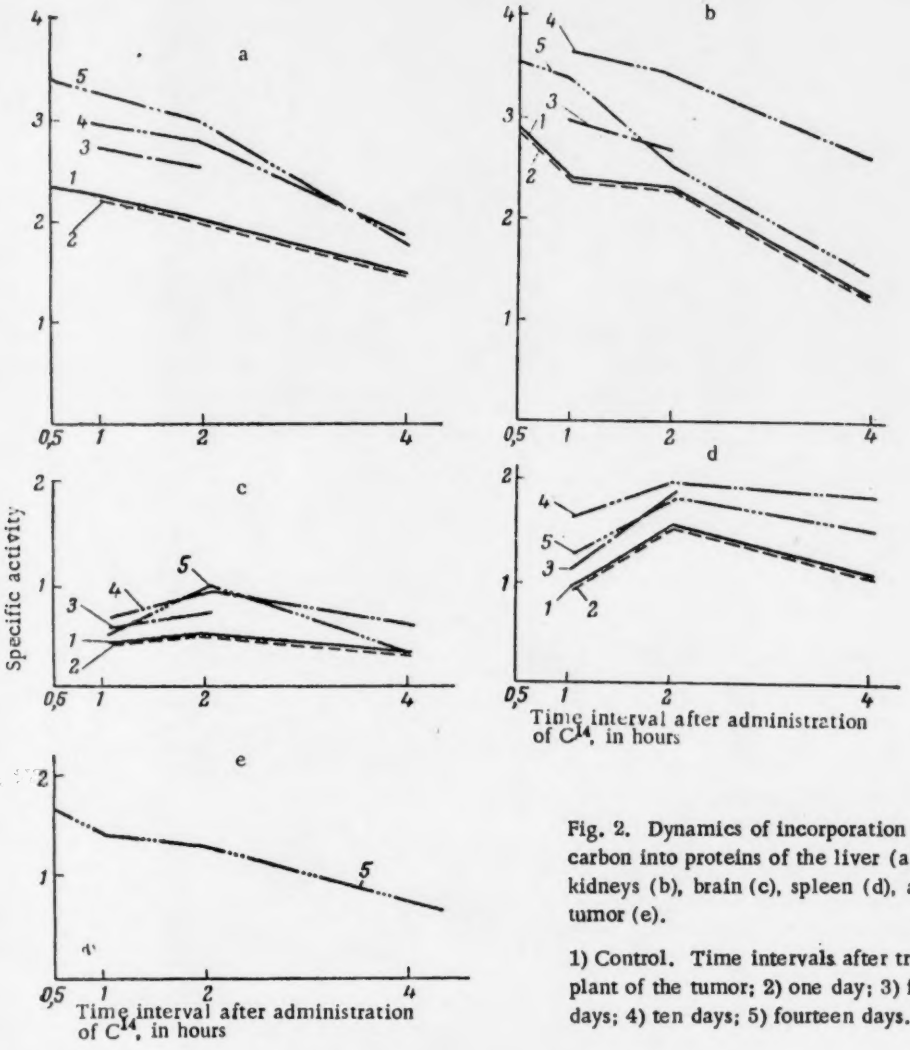

Fig. 2. Dynamics of incorporation of carbon into proteins of the liver (a), kidneys (b), brain (c), spleen (d), and tumor (e).

1) Control. Time intervals after transplant of the tumor; 2) one day; 3) five days; 4) ten days; 5) fourteen days.

The test organs were taken from the mice on the first, fifth, tenth and fourteenth days following the beginning of the transplant; 17 animals were taken on the first day, 20 on the fifth, 34 on the tenth and 47 on the fourteenth (equal number of control and experimental animals).

The dynamics of incorporation of carbon into the proteins of the liver (a), kidneys (b), brain (c) and spleen (d) is shown in Fig. 2.

A day after the transplant, the incorporation of carbon into the protein fraction of the liver in the control and the experimental animals proceeded at the same rate. From the fifth day and during the next 14 days, the

incorporation of carbon in the experimental animals was noticeably different from the controls. This difference consisted of a greater intensity of incorporation of carbon into the proteins. Similar results were obtained also in the study of the spleen (d) and the kidneys (b). However, it was not difficult to note a significant difference in the dynamics of incorporation of carbon into the proteins of these organs.

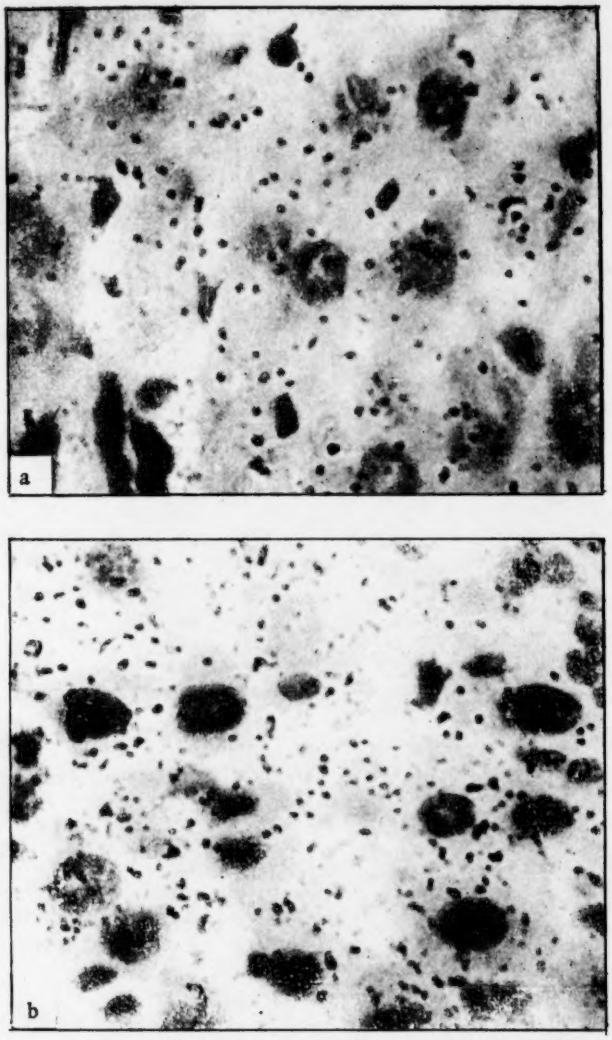

Fig. 3. Histoautoradiogram of the liver of control mice after one hour following the administration of radioactive carbon:
A) control mice; B) experimental mice ten days after the tumor transplant. Black dots) traces of paths of β-particles from C^{14}.
Exposure: 30 days.

As it is evident from Fig. 2 d and b, the greatest differences between the control and the experiment in these cases are observed on the tenth day after the transplant. By the fourteenth day, when the tumor reaches large dimensions, the rate of incorporation of carbon approaches that of the control. Consequently, the difference in the dynamics of the incorporation of carbon into the proteins of the liver of experimental animals increased continuously, the maximum difference in the spleen and the kidneys declined on the tenth day and then

this dropped with the development of the tumor. The rate of incorporation of carbon in the brain tissue (c) proceeded at a much lower level than in the other organs and practically did not differ from the control animals. Data on the incorporation in the tumor tissue are given in Fig. 2d.

It is possible to see in the resulting material, in addition to this, that the dynamics of incorporation of carbon dioxide into the proteins of various tissues proceeds differently. The maximum of incorporation in the liver and the tumor occurs within the first thirty minutes; in the spleen and the brain – by the second hour, while in the kidneys it is observed as two maxima: the first arising in 30 minutes, the other after two hours. Evidently, this is explained by the specificity of metabolism in these organs.

In order to be convinced further that the incorporation of carbon dioxide proceeds differently in the organs of the animal organism with a developing tumor than it does in controls, we set up some experiments on the determination of the the amount of exhaled radioactive carbon dioxide. For this purpose and immediately after the introduction of the radioactive bicarbonate, the control and the experimental animals were placed into different, but equal in respect to the volume of contained air, glass covers (domes) in which the carbon dioxide was absorbed by caustic alkali. (In order to eliminate the inaccuracy in the accounting for the exhaled carbon dioxide, the domes were changed in the various series of experiments). Equal amounts of the alkaline solution were taken for the tests after 30 and 60 minutes from the control and the experimental animals.

Change in Rate of P^{32} Incorporation in Nucleoprotein Fractions from the Liver, Kidneys, and Spleen of Animal Carriers of Tumors (in % with respect to the controls)

Time after tumor implantation	Liver	Kidneys	Spleen
1 day	125	87	65
5 days	131	100	80
10 days	108	102	70

As a result of a large number of observations it was found that, beginning with the fifth day after the transplant of the tumor, the amount of liberated carbon dioxide in the experimental animals was smaller than in the controls. By the tenth day this difference reached an average of 20%. This means that in an animal, during the development of a tumor in it, the amount of retained carbon dioxide is 20% greater than in the controls. Similar data were obtained also in the study of the dynamics of incorporation of carbon (the difference between the controls and the experiment was also approximately 20%). In some cases we made histoautoradiographic studies of sections of the organs. Prior to taking the autoradiogram, the sections were placed into a Warburg apparatus for the removal of free carbon dioxide.*

The histoautoradiograms of the sections of the liver of control (A) and experimental (B) animals after ten days following the transplant and after one hour after administration of labelled carbon are given in Fig. 3. As is evident from Fig. 3, the amount of runs made by the β-particles of C^{14} in the liver of experimental animals is greater than that in the liver of the controls.

Thus, the various methods of study used by us permit us to regard with a considerable degree of definition that it is proven that there exists a difference in the rates of incorporation of carbon dioxide into the protein fractions of the liver, the spleen and the kidneys of the experimental and the control animals.

Owing to the close connection of protein metabolism with that of the nucleoproteins, it was natural to follow up the state of this metabolism under analogous experimental conditions.** For this purpose, we set up the studies on the determination of the rate of renewal of the nucleoproteins, with the aid of radioactive phosphorus.

Both the experimental and the control animals were given, at the same time after the transplant, sodium phosphate with P^{32} amounting to 10 μcurie per mouse with 0.02 mg of phosphorus carrier. Two hours later, the mice were killed and nucleoproteins were preparatively isolated from the liver, the spleen and the kidneys, after which phosphorus was determined in these by the Fiske-Subbarow method. The specific activity was calculated per 1 mg of P [31]. In all, 75 control and 75 experimental animals were used for the experiment.

The data on the rate of incorporation of P^{32} into the nucleoproteins of the liver, kidneys and spleen of the animals, in which tumors had been transplanted, are given in the table in percent relative to the healthy specimens. As is evident from the table, one day after the transplant the nucleoprotein metabolism in the liver increases and reaches its maximum (31%) by the fifth day. By the tenth day, when the tumor appears in the animal's organs,

* This part of the work was performed jointly with E. I. Shcherban'.

** E. I. Orlova took part in this section of the work.

the rate of renewal of nucleoproteins comes back to normal. The rate of nucleoprotein metabolism in the kidneys declines somewhat (by 13%) during the first days and later remains equal to the controls. In the spleen, the nucleoprotein metabolism is considerably depressed from the first to the tenth day. The rate of renewal of nucleoproteins in the tumor tissue is approximately two-three times smaller than in the organs of animals used for controls and afflicted with the tumor.

DISCUSSION

Our experimental material testifies that within the first day after the implantation of the tumor (Crocker sarcoma) significant changes occur in the nucleoprotein metabolism in the organs of the animals. A disturbance of the protein metabolism also occurs five days after the transplantation. Here, in each organ, these changes occur differently.

Doubtless, it is difficult at this time to answer the question about the causes of these disturbances and about their significance in the pathogenesis of the tumor growth. We wish only to express some of our ideas.

As long ago as 1948 we succeeded in isolating from a rat sarcoma a nucleoprotein complex which in its physicochemical [7] and immunological properties [32] differed from the nucleoproteins of the normal tissue. This gave us the right to suppose that a peculiar, uniquely possessed nucleic acid exists in the tumor cells, this acid being able to form complex compounds with protein, such compounds being characteristic of only the tumor tissue. According to our ideas, the structure of such a nucleic acid should be less complex than that of the nucleic acids isolated from the highly differentiated cells. In recent years there has appeared a series of papers which to some degree experimentally confirm this point of view. Thus, Butler and coauthors [2], Werkheiser and Visser [3], and Schmitz and coauthors [4] have detected differences in the content of purine and pyrimidine bases in nucleic acids isolated from the tumor tissue. Steckerl [5] succeeded in isolating a ribonuclease from the cells of mouse ascytic cancer, this enzyme being incapable of cleaving yeast ribonucleic acid. The author notes the formation of RNA in the tumors, this RNA being esterified at the 2nd and 3rd atoms of ribose. Gizatullina [33] isolated from some microbes a desoxyribonuclease which cleaves DNA isolated from the ascytic cancer and which is inactive against DNA of normal tissue.

The experimental material cited above in this work showed that significant changes occur in the nucleic metabolism under the influence of the tumor implantation after but a day, these changes being evident in the liver and the spleen of the animals. Changes in the protein metabolism of these animals set in considerably later.

Consequently, long before the development of the tumor, changes in the nucleic metabolism occur first in some of the organs of the animal and then, only as a consequence of this disturbance, changes occur in the protein metabolism also.

Naturally, a question arises as to why does the disturbance of nucleic metabolism occur in animals afflicted with the tumor and does this disturbance appear to be that primary link on which the cancerogenic substances actually operate ?

Doubtless, this question is very difficult to answer so far. However, we believe (based on the material existing in the literature and also on our own observations) that the distortions in the nucleic and protein metabolism occur under the influence of the disturbance of the process of tissue respiration in the cells of the animal organism, caused by cancerogenic substances which differ in their nature, (chemical and physical agents being considered).

In this respect we share completely the view point of Warburg and of a number of other investigators, who regard that the distortion of tissue respiration occurs within the tumor cell. Warburg [14] supposes that the respiration energy is replaced by the glycolysis energy in the tumor cell. Owing to the deficit of fermentation energy, the highly differentiated normal cells are transformed into undifferentiated ones with disordered growth, characteristic of the cancer cells. Holzer, Hean and Petter [15] support Warburg's hypothesis to the effect that if one deprives a tumor cell of its fermentative ability, the tumor cell will cease to exist. Kertesz and Albano [16] consider that the enhanced glycolysis observed by them in tumor cells is the result of a disturbance in the electron-transfer system. Kit [11] cites a number of convincing data in favor of direct oxidation of glucose in the tumor cells. A number of authors [18, 34] have shown that the cancer cell is resistant to the action of cyanides.

Our experiments with labelled carbon dioxide also testify of the alteration of tissue respiration. As indicated above, beginning with the fifth day following the transplant there is observed in the animal organism a retar-

dation of elimination of free carbon dioxide at the expense of increased incorporation of it into organic substances of organs and tissues, which may take place as a result of alterations which occur in the citric acid cycle in which carbon dioxide takes an active part. Thus, the distortion of the normal respiration, connected with carbon dioxide participation, indicates indirectly a damage to tissue respiration which brings with it a distortion of biological oxidation of organic substances and, in the first place, nucleoproteins.

Consequently, long before the appearance of the tumor, a damage sets in in some cells of the animal organism under the influence of cancerogenic agents, this damage being directed to suppression of some links in tissue respiration, as a result of which the metabolism in these cells proceeds under a low energetic level. This, in turn, leads to a change of highly differentiated cells, which require a considerable amount of energy for their development, into slightly differentiated cells.

Such cells are capable only of disordered division, which does occur in the tumors.

SUMMARY

The dynamics of incorporation of carbon into protein substances of the liver, kidneys and spleen of animals which are carriers of a tumor, differ considerably from the controls. This difference sets in by the fifth day following the tumor transplant.

The change in the nucleoprotein metabolism occurs in the liver and the spleen during the first day after transplantation of Crocker sarcoma to the mice.

The weight of the organs of animals with a tumor is greater than that in the control animals.

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THE INFLUENCE OF THYROID-GLAND HORMONES ON GLUCOSE METABOLISM

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It is known that the thyroid-gland hormones exert a considerable influence on carbohydrate metabolism. However, the results of investigations of this problem and their interpretation by the various authors often fail to coincide [1–3].

Mirski and Broh-Khan [4] showed in experiments with eviscerated animals that thyroxin raises the consumption of oxygen and utilization of glucose. Introduction of thyroxin into rats accelerates the decomposition of glycogen in the brain, stimulates the phosphorolysis processes, those of glycolysis and metabolism of pyruvic acid, and raises amylase activity [5]. These data find a confirmation in the work by Milcu and co-workers [6] who showed that introduction of thyroxin stimulates the cleavage of glycogen, evidently, at the stage of anaerobic glycolysis.

In humans and experimental animals with enhanced thyroid-gland function there is observed a hyperglycemia, glycosuria, raised glycemic curve and lowered sensitivity to insulin [2, 7, 8]. The lowered tolerance to sugar in thyroid-gland hyperfunction is explained by Shpiner [9] by the toxic action of thyroxin on the β-cells of the pancreas; Cramer [10] by disturbance of glycogen-forming function of the liver. In Althausen's opinion [1], thyroxin accelerates the assimilation of sugar from the intestine and it is, specifically, this (and not the absence of the ability of the organism to assimilate glucose) that is the cause of the enhanced glycemic curve after peroral administration of glucose. There are also some data [2, 11] which indicate that the form of the glycemic curve in thyroid-gland hyperfunction depends also on the state of the nervous system.

In the present work we give the results of studies on the influence of thyroxin, thyroidectomy, as well as thyrotropic hormone, on the carbohydrate metabolism and on the tolerance toward sugar, on rabbits.

METHOD

The experiments were run with rabbits weighing 2.5–3.5 kg, after 30 days and 10 months following thyroidectomy. Part of the animals received 0.25 mg/kg of body weight doses of thyroxin daily over ten days. Normal rabbits of the same weight served as the controls. All rabbits had a standard diet; during the experiment they were weighed periodically and a determination of the content of cholesterol and organically bound iodine in the blood was run. The completeness of the removal of the thyroid gland in the thyroidectomized animals was checked by the examination of the throat area and by measurement of accumulation of I^{131}. The assimilation of glucose and the tolerance of the organism to sugar, among the experimental animals, were determined after an intravenous introduction of 0.5 g/kg of body weight of glucose. Prior to the experiment, the rabbits did not receive any food for 16 hours. The glucose concentration in the blood was determined prior to the introduction and after 10, 20, 45, 60, 75 and 90 minutes following the administration of glucose.

From the data of these determinations, we constructed the glycemic curves on semilogarithmic paper. Glucose concentration varied in direct proportionality to time. From the slope of these curves, we determined the rate of assimilation of glucose and the tolerance to it. For expressing the rate of assimilation of glucose we used the "coefficient of glucose utilization" determined by the method described in the works of Amatuzio et al [12], Connard [13] and Macho and Licko [14].

RESULTS

It is evident from the data in Table 1, that in rabbits, following 30 days after thyroidectomy, the blood-sugar level after an intravenous administration of glucose is lowered slowly and for this reason the slope of the glycemic curve is characterized by shallowness. The value of the coefficient of glucose utilization is considerably lower than before the operation. Introduction of thyroxin (0.25 mg/kg of body weight) over 10 days leads only to a rather insignificant and uncertain rise of the glucose-utilization coefficient. The resulting data indicate that the removal of the thyroid gland greatly lowers the degree of assimilation of glucose, while the experimental thyrotoxicosis practically does not alter the tolerance to glucose and its assimilation in comparison with the norm.

TABLE 1

Change of the Coefficient of Glucose Utilization in Rabbits After the Removal of the Thyroid Gland and Subsequent Administration of Thyroxin

State of animals	No. of animals	Coefficient of glucose utilization	p	Δ of coefficient of glucose utilization	p
Prior to thyroidectomy	8	1,14±0,07	—	—	—
30 days after thyroidectomy	8	0,54±0,09	<0,001	—0,60±0,10	<0,001
Prior to admin. of thyroxin	7	1,16±0,18	—	—	—
10 days after administration of thyroxin (0.25 mg/kg)	7	1,29±0,25	>0,1	+0,13±0,13	>0,1

TABLE 2

Change in the Coefficient of Glucose Utilization in Normal Rabbits and 10 Months After Thyroidectomy, Before and After Administration of Thyrotropic Hormone

State of animals	Number of animals	Coefficient of glucose utilization	Δ of coefficient of glucose utilization	p
Normal, prior to administration of thyrotropic hormone	4	1,10±0,16	—	—
After administration of thyrotropic hormone	4	1,47±0,05	0,36	0,001
10 months after thyroidectomy, prior to administration of thyrotropic hormone	4	0,72±0,15	—	—
After administration of thyrotropic hormone	4	1,20±0,13	0,47	0,02

Data on the influence of the thyrotropic hormone on the rate of assimilation of glucose are given in Table 2. The determination of assimilation of glucose was run after its intravenous administration, before and after 4 hours following the administration of a pure preparation of thyrotropic hormone (0.1 mg/kg of body weight). It follows from the cited results, that the rate of assimilation of glucose increased, after the administration of the thyrotropic hormone, both in the normal animals and in animals after 10 months following a thyroidectomy.

DISCUSSION OF THE RESULTS

It follows from the results of our experiments that the assimilation of glucose in the tissues and the sugar tolerance after the removal of the thyroid gland are both depressed. The cause of the lowering of this assimilation is evidently the deficiency in the thyroid-gland hormones in the organism and the absence of their stimulating effect on metabolism.

We found a small rise of glucose assimilation after administration of thyroxin. In not a single animal did we observe an elevated glycemic curve, i. e.,lowered sugar tolerance. Similar results have been obtained in the work by Milcu [11] and Amatuzio [15]. This gives a basis for the consideration that the elevated glycemic curve after a sugar loading with an enhanced thyroid-gland function is actually created by an accelerated absorption of glucose from the intestines, as was supposed earlier [1].

We observed a rise in glucose assimilation in healthy and thyroidectomized animals following the administration of the thyrotropic hormone. The secretion of hormones of the thyroid gland, which accelerate the metabolism of glucose in the organism, is raised in normal animals after the administration of thyrotropic hormone. The action of the thyrotropic hormone on animals with removed thyroid gland is evidently a consequence of a direct action of this hormone on glucose metabolism. The substance of this action is still unknown and it is also possible that the thyrotropic hormone activates the extrathyroidal tissues which secrete the thyroidal hormones.

SUMMARY

The assimilation of glucose and sugar tolerance are lowered in rabbits after thyroidectomy.

Administration of thyroxin causes an insignificant and uncertain rise of the glucose-utilization level. After the administration of the thyrotropic hormone, there is observed a rise in glucose assimilation in both the normal and the thyroidectomized rabbits.

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STUDIES OF PAPER ELECTROPHORESIS. COUNTERCURRENT ELECTROPHORESIS IN THE SYSTEM OF ENZYME–SUBSTRATE

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The technique of paper electrophoresis has occupied a secure place in the research and clinical laboratories for the separation of proteins, nucleic acids and a number of other compounds. A special interest exists in the electrophoretic analysis of blood-serum proteins.

Usually the technique of serum electrophoresis consists of the deposition of 0.05–0.1 ml of serum onto a strip of filter paper saturated with a veronal buffer, spreading of the protein fractions by means of potential difference, fixation of the protein fractions and their subsequent staining with one of the protein dyes: Bromophenol blue, Azocarmine, fuchsine, amido black, benzidine, etc.[1–4]. With this technique of electrophoresis, the experimenter lacks the possibility of determining the rate of the movement of the protein front during the experiment, since the protein remains invisible prior to the instant of development. In addition, after running electrophoresis by the generally accepted technique and after staining of the electrophorogram, one cannot bring out the qualitative peculiarities of individual protein fractions among which the greatest interest is caused by the biocatalytic activity possessed by many proteins.

Numerous attempts to apply electrophoresis to the clarification of the link between the enzymic activity with some particular protein fraction have been described in the literature [5–15]. For the establishment of localization of the enzyme, the workers most frequently used the method of elution of individual sections of the electrophorogram and the determination of the specific activity of the resulting eluates by the usual methods.

By using this method, Jermin and his co-workers [5, 11] were able to establish the presence, in extracts of Aspergillus oryzae, of two fractions which possess an esterase activity and six fractions which show the amylase activity. Tognie and Meier [6] discovered an esterase activity in the β-globulin fraction, after electrophoresis of horse-blood serum. Delcourt and Delcourt [7], in studying the mouse-blood serum, found a clear maximum of esterase activity corresponding to the localization of α- and β-globulins.

Sumtsov [8], studying the lipase activity of protein fractions of breast milk, came to the conclusion, after their electrophoretic separation on paper, that the enzyme being examined is in the γ-globulin fraction.

Baker and Pelligrino [9] used for the same purpose the technique of spraying the electrophorograms with solutions of substrate, incubation in a thermostat, and subsequent spraying with a developer for the cleavage products of the substrate. The solution of tributyrin was used for the indication of lipase, the above substance acting as the substrate, while a solution of the universal indicator was used as the developer; for the detection of esterases, a solution of butyrylcholine was used instead of tributyrin. As a result of this, the aliesterase and cholinesterase activities were found in the same protein fraction localized between the α- and β-globulins; the phosphatase activity showed two maxima, corresponding to the α- and γ-globulin fractions; amylase was concentrated in the γ-globulin fraction.

Mills and Smith [10] used the method of contacting the electrophorograms with strips of filter paper, which had been saturated with the substrates which form colored products after decomposition, for the showing of the location of the enzyme fractions. Cohen and Warring [14] determined the location of cholinesterase of human blood serum by the deposition of agar plates, in the composition of which acetylcholine had been introduced along with Phenol red, onto the resulting electrophorograms (on paper or on starch) [15].

Thus, attempts to use electrophoresis on paper for the detection of location of the individual enzymes have been reflected in a number of studies. However, the running of the electrophoresis and the determination of the enzymic acitivity in the various regions of the electrophorograms had been run successively, as two separate procedures. The latter circumstance complicated the problem in a majority of cases and made its resolution less accurate.

We undertook an attempt to develop a new modification of this method, suitable for the simultaneous examination of enzyme properties of protein fractions of blood serum and their electrophoretic separation.

The work consisted of two stages. First we developed the methods which permitted one to follow the rate of motion of the protein fractions during the electrophoresis proper, after which we studied the possibility of detection of converging reactions between the protein fractions having enzymic activity and the corresponding substrates during the process of electrophoresis.

The preliminary experiments confirmed our supposition that the introduction of sufficient concentrations of some indicators into the usual veronal buffer, or other buffer systems, permits one to follow the motion of the protein front. The indicator-buffer mixtures suitable for these purposes should have the following properties: they should give clearly distinct color changes in the pH interval of the buffer system used and the blood serum (which should not coincide); they should be water soluble; neither the indicators nor the components of the buffer mixture should produce any hindering action on the enzyme being studied, at the concentrations used.

TABLE 1

The Character of Coloration of Electrophorograms and of The Rate of Motion of The Protein Fractions in Running Electrophoresis With Various Indicator-Buffer Solutions. Electrophoresis was run with current intensity of 0.25 mA/cm

Buffer	Indicator	Background color of paper	Color of stripe corresponding to serum disposition	Character of stripe corresponding to the front of motion of protein during electrophoresis	Rate of motion of electrophoretic front in cm (from the start line) in:			
					1 hr	5 hr	10 hr	15 hr
Borate	Bromothymol blue	Light blue	Yellow	Wide, white stripe	2	6	10,5	15
»	Phenol red	Raspberry red	Weak pink	Narrow stripe with weak pink color	2	6	10,5	14,5
»	Bromothymol blue + Phenol red	Violet	» »	Narrow strip with weak pink color with a following bright red stripe	2	6	11,0	15
Veronal	Bromothymol blue	Light blue	Yellow	Wide white stripe	1,5	4,5	8	12

The studies made by us showed that such demands are met in a definite measure by the borate, veronal-medinal and phosphate buffers and the following indicators: Bromothymol blue, Cresol red and Phenol red – from which we made up the electrophoretic indicator-buffer solutions. Satisfactory results were obtained with solutions of the following composition.

I. Bromothymol blue (borate solution). 500 mg of crystalline Bromothymol blue indicator was dissolved by rubbing in an agate mortar with 11.4 ml of 0.1 N sodium hydroxide solution and this was transferred to a volumetric flask of 1 liter capacity. To this was added 91.4 ml of 0.1 N sodium hydroxide solution and 250 ml of 0.1 M boric acid in 0.1 M potassium chloride solution. The flask contents were adjusted to the mark with distilled water. The solution had a dark blue color and pH 8.6.

II. Phenol red (borate solution). 500 mg of crystalline Phenol red was mixed by rubbing in agate mortar with 14.2 ml of 0.1 N sodium hydroxide solution. Then the steps were as for the preparation of mixture I. The solution had a dark red color and pH 8.6.

III. Bromothymol blue or Phenol red (veronal solutions). The solutions of Bromothymol blue or Phenol red, prepared as indicated above, were dissolved in 1 liter of the usual veronal-medinal buffer. The latter was prepared by solution of 8.712 g of veronal, 1.893 g of sodium hydroxide, 6.476 g of sodium acetate and 60.0 ml of 0.1 N hydrochloric acid in distilled water with adjustment of the resulting volume to one liter. In addition, we studied the borate-buffer mixtures, including the combination of indicators prepared by mixing equal volumes of solutions I and II.

The electrophoresis was run in the apparatus of the type LKB on strips of filter paper with dimensions of 40 x 4 cm, preliminarily saturated with the indicator-buffer mixtures being tested. After the removal of excess liquid, the strips were placed in the chamber of the apparatus and were kept with closed lid for 15 minutes. Then, the blood serum was deposited with calibrated pipettes in the amount of 10 or 20 μliter in the form of a uniform line located 80 mm from the middle of the strip in the direction of the cathode. The location of the deposition of the serum was shown on the background of the paper stained by the indicator in the form of a clearly distinguishable light stripe.

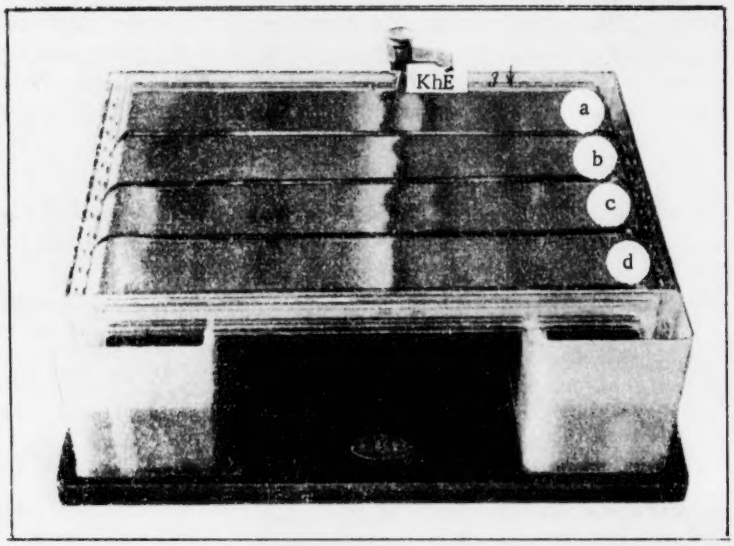

Fig. 1. General view for running the countercurrent electrophoresis of choline sterase of human blood serum with acetyl- and butyrylcholines with utilization of a mixture of borate buffer and bromothymol blue.
a) Countercurrent electrophoresis with butyrylcholine; b) control; electrophoresis of blood serum; c) countercurrent electrophoresis with acetylcholine; d) countercurrent electrophoresis with butyrylcholine and serum previously treated with Chlorophos. 1) Bright-blue stripe corresponding to the albumin fraction; 2 and 3) increase of intensity of blue color corresponding to the globulin fractions; KhE) yellow stripe especially pronounced in countercurrent electrophoresis with butyrylcholine. The line of deposition of the serum is shown by the arrow.

Depending on the predetermined conditions, the electrophoresis was run at current strengths from 0.25 to 0.5 ma per cm of the front and with potential of 120 to 250 V.

Soon after switching on the current, the light stripe corresponding to the location of deposition of the serum, widened somewhat and began to move gradually toward the anode. For solutions in which Bromothymol blue was used as the indicator, the stripe became white instead of yellow. After the white stripe, came a stripe colored intense blue green, which indicated an accumulation of the indicator. In a number of cases, especially with the use of the borate solutions, it was possible to note in the distribution zone of the protein fractions, some differences in the color intensity.

The character of coloration of the electrophorograms and of the rate of motion of the front in running the electrophoresis with various buffer-indicator solutions are shown in Table 1.

For clarifying the causes for the formation of the indicated stripes which reflect local pH changes and changes in indicator concentration, we studied in parallel on the same electrophorograms the location of the protein fractions after their fixation and staining with Bromophenol blue by the usual method. For this purpose, the electrophorograms taken at various intervals, were dried in a drying oven at 100° and were cut lengthwise into two equal parts. One of these was placed for 10 minutes into a dish with 0.05% Bromophenol blue solution in 1% mercuric chloride solution along with 2% acetic acid solution; the excess dye was washed off in 0.5% acetic acid solution and the strip was dried.

A comparison of the location of the stripes on the developed and undeveloped electrophorograms indicates that the light stripe observed during the electrophoresis characterizes the forward boundary of the front of moving and most-mobile protein fraction of the blood serum – albumins. The dye concentration in the adjoining zone corresponds to the albumin fraction proper. The location of the subsequent protein fractions is shown considerably less distinctly in the undeveloped chromatograms.

Let us express some preliminary considerations about the possible mechanism of formation of the visible stripes during the running of electrophoresis with the indicator-buffer mixtures. The origin of the white stripe which corresponds to the frontal boundary of the moving front of albumins, evidently characterizes a sharp drop of indicator concentration. This local decline in the indicator concentration may be understood when one considers that during the deposition of serum there occurs a local drop in pH which is detectable by the indicator color change. Here, in accord with the chromophore-ionic theory of indicators, those of the sulfophthalein structure (Bromothymol blue and Phenol red), being weak acids, pass from the dissociated into the undissociated form. Simultaneously they undergo an intramolecular rearrangement which is accompanied by the transformation of the quinophenolate anion into the sulfophthalein molecule. As a result, not only is color change observed, but so is the loss of ability to move in an electric field.

Deposition of the protein onto a paper strip saturated with the indicator-buffer solution, leads to the appearance of two forms of the indicator, colored differently from each other, one of which (blue for Bromothymol blue or red for Phenol red) moves toward the anode in an electric field, while the other (yellow for both indicators) remains in the same place. This leads to a zonal drop of indicator concentration at the frontal boundary of protein movement and to an increased indicator concentration in the neighboring zone where the indicator ions, moving toward the anode, catch up with their undissociated forms.

It is necessary during the treatment of this phenomenon to consider the differences in rates of motion on filter paper, of relatively small indicator molecules in comparison with the protein macromolecules. The indicator ions undoubtedly move considerably more rapidly than does the protein. A direct measurement of the rate of motion of the indicators used by us under similar experimental conditions shows that the indicators move considerably more rapidly than the proteins in an electric field. Consequently, the indicator ions should have outrun the protein anions. However, this does not happen since on reaching the location zone for the protein, the indicator passes into the undissociated form and loses mobility. As a result of this, an increase of indicator concentration occurs which is proportional to the buffer properties of the protein. Thus, the local change of pH on the electrophorogram during the running of electrophoresis with indicator-buffer mixtures should, as a rule, be accompanied by local accumulation of the indicator. This principle may evidently find some application in the electrophoretic analysis of substances having a considerable buffer capacity.

The method of electrophoresis developed by us for blood serum on paper saturated with the indicator-buffer mixtures permits one to make electrophoresis visible and allows one to follow the progress of the motion front of the protein. At appropriate concentrations of the serum, the motion of the albumin fraction is revealed with a high degree of contrast, which permits us to recommend this tecnique for the demonstration of principles of electrophoresis on paper. Especially satisfactory for the purposes of lecture demonstrations may be the use of combined indicator-buffer mixtures (a mixture of Bromothymol blue and Phenol red), with which a white and a bright red stripe appear on a violet background in the process of electrophoresis (Fig. 1), while in the case of a converging reaction in the system of enzyme-substrate (see below) a lemon-yellow stripe appears.

The use of indicator-buffer solutions in no way affects the distinctness of location of the protein fractions in their subsequent staining by the usual methods and does not complicate the technique of running the electro-

phoresis and treatment of the electrophorograms. At the same time, the introduction of indicators into the buffer solutions undoubtedly provides certain conveniences for the work. It allows one to distribute the serum on the starting line more uniformly, under the control of the eye, to control the rate and the uniformity of motion of the protein in various paper strips during the course of electrophoresis (and because of this to eliminate, in time, any possible technical errors), to follow to a certain extent the quality of the buffer solutions and to attain more standard conditions for running the experiment.

TABLE 2

Location of Cholinesterase Activity and Protein Fractions of Blood Serum In Countercurrent Electrophoresis

Blood serum		Distance of the fraction from site of deposition, in cm				
		Albumins	Globulins			Cholinesterase
			α	β	γ	
Human:	developed	12,6	8,8	7,3	4,5	—
	undeveloped	12,4	—	—	—	8,8
»	developed	11,8	8,5	7,2	4,6	—
	undeveloped	11,5	—	—	—	8,5
»	developed	12,0	8,4	7,0	4,4	—
	undeveloped	12,3	8,8	—	—	8,8
»	developed	12,2	8,8	7,3	4,9	—
	undeveloped	12,2	—	—	—	8,8
»	developed	11,8	8,7	7,3	4,7	—
	undeveloped	11,9	—	—	—	8,8
»	developed	12,2	8,8	7,3	4,6	—
	undeveloped	12,2	—	—	—	8,8
»	developed	12,5	9,1	7,6	4,9	—
	undeveloped	12,5	9,1	7,7	5,2	9,2
Mouse:	developed	12,9	10,4	7,5	5,5	—
	undeveloped	12,8	10,0	7,3	4,8	8,5
»	developed	13,3	10,7	7,8	5,6	—
	undeveloped	13,2	—	—	—	10,0
»	developed	12,8	10,2	7,4	5,2	9,6
	undeveloped	12,9	—	—	—	9,5
»	developed	12,7	10,5	7,8	5,7	
	undeveloped	12,6	—	—	—	10,0
						10,0
Guinea pig:	developed	12,3	10,5	8,8	4,6	—
	undeveloped	11,9	—	—	—	7,9
»	developed	12,2	10,4	8,5	4,3	8,2
	undeveloped	11,8	—	—	—	7,7
»	developed	12,4	10,5	8,9	4,9	8,8
	undeveloped	12,7	—	—	—	8,6
»	developed	12,3	10,6	9,0	5,3	—
	undeveloped	12,3	—	—	—	8,5

However, the advantages of the indicator-buffer mixtures were shown to the greatest degree during the solution of the second problem before us – the use of the electrophoretic method on paper for identification of the protein fractions of the serum which are the carriers of esterase activity. We attempted for this purpose to run converging reactions between the enzyme and the substrate by means of paper electrophoresis. The specific substrate in this case should be forethought as the indicator which detects the protein fraction having the specific activity of an enzyme. Actually, the protein particles acquire a negative charge in buffer solutions with an alkaline reaction and move toward the anode in an electric field. On the other hand the molecules of choline esters, being quaternary ammonium compounds, have a positive charge and move toward the cathode in the electric field. Consequently, in the system of cholinesterase-choline esters, the realization of a countercurrent electrophoresis of the enzyme with the substrate is possible. Insofar as a separation of the protein fractions occurs during the process of electrophoresis, one may suppose that the cleavage of the substrate would be revealed in the zone of location of the fraction which is the carrier of the specific enzymic activity.

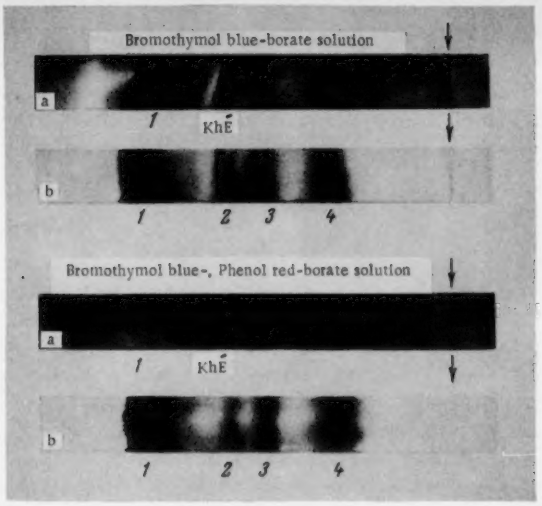

Fig. 2. Electrophorograms of human-blood serum, obtained by the use of various indicator-buffer mixtures:
a) uncolored electrophorograms; b) colored electrophorograms. 1) albumin fraction; 2, 3 and 4) fractions of globulins; KhÉ) yellow stripe on pale-blue background in case of indicator-buffer mixture with Bromothymol blue or on violet-red background in case of indicator-buffer mixture with Phenol red and Bromothymol blue.

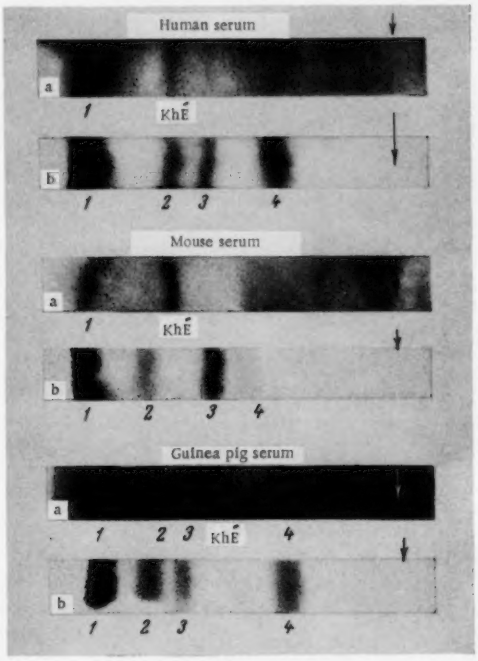

Fig. 3. Colored and uncolored electrophorograms of blood serum of some mammals.
a) uncolored electrophorograms; b) colored electrophorograms. 1) albumin fraction; 2, 3 and 4) fractions of globulins; KhÉ) yellow stripe corresponding to cholinesterase. The deposition line is shown by arrow.

In running the countercurrent electrophoresis, the technique remained the same as in the work with the indicator-buffer solutions, but a solution of the substrate of acetyl- or butyrylcholine was additionally deposited on the paper as a transverse stripe at the side nearer the anode. The 5% substrate solution and the undiluted blood serum in the amount of 10–20 μ liter were deposited symmetrically on both sides of the midpoint of the paper strip. The appearance of the electrophorograms at the beginning of the experiments with countercurrent electrophoresis differed only by the presence of the additional whitish stripe which corresponded to the site of deposition of the substrate. This stripe was not distinct in the usage of a basic indicator-buffer solution as the substrate solvent. However, after 6–7 hours following the switching-on of the current, a narrow but clearly visible greenish stripe appeared 1.5–2 cm from the boundary of the albumin fraction from the cathode side and this stripe soon acquired a yellow coloration. After 5–10 minutes this became bright yellow, then widened gradually, acquired a more saturated color in the center which approached orange. The yellow stripe which characterized a local lowering of pH, occurred only on those electrophorograms onto which both the serum and the choline ester had been deposited simultaneously. The localization of the stripe and the time of its appearance in parallel experiments were distinguished by considerable constancy. The appearance of the stripe was never noted in experiments in which only the serum or only the substrate were deposited.

The location of the stripe did not depend on the nature of the choline ester (acetylcholine, butyrylcholine, thiobutyrylcholine), but for the ester of butyric acid, the stripe appeared somewhat sooner and had a more intense color.

Evidently, the appearance of the yellow stripe may be connected with a local pH change occurring at the site of meeting of the choline ester with the cholinesterase and the accumulation of hydrolysis products which have an acid reaction. Some additional confirmations of this treatment were secured in experiments with repression of the cholinesterase activity by means of specific inhibitors. For this purpose, the blood serum was treated with aqueous solutions of proserine and phosphorus containing insecticides (Mercaptophos and Chlorophos) in doses which insure a complete inhibition of cholinesterase activity. After 30 minutes the treated and the untreated (with inhibitors) serum were deposited on the starting lines of electrophorograms and the current was switched on. As a result, we observed sharp differences in the intensity of the yellow color formed at the site of meeting of the enzyme with the substrate. The width and the intensity of the yellow-colored stripes formed in experiments with the inhibitors were much lower than in the control electrophorograms.

It is interesting to note the appearance of action of cholinesterase on electrophorograms in the presence of high concentrations of inhibitors which insure a complete inhibition of the enzyme in checks of the activity by a chemical method. This phenomenon must be evidently explained by a partial restoration of enzyme activity owing to the separation of loosely linked residues of inhibitor molecules during the run in the electric field.

The question of the correspondence of the cholinesterase activity in a definite protein fraction may be resolved by a comparison of the colored and uncolored electrophorograms. Here it was shown that in case of a simultaneous deposition on the paper of the serum and of the choline ester solution, the appearance of the yellow stripe occurs prior to the moment of a sufficiently distinct separation of the protein fractions. Later, the deposition of enzyme and substrate solutions was done at different times. The blood serum was deposited first at the cathode side and the current was switched on. After the attainment of the required degree of separation of the protein fractions, the solution of butyryl- or acetylcholine was deposited at the anode side. After a distinct revelation of the yellow stripe, the electrophoresis was stopped and the electrophorograms were dried in a drying oven. Each electrophorogram was cut lengthwise into two equal halves; one of these was subjected to the standard staining, while a comparative study of the location of the protein fractions was run on the other.

The results of the distribution on electrophorograms on the cholinesterase activity and of the various protein fractions of blood serum are shown in Table 2. For making a more exact characterization of the translocation of the various fractions, we give in this table the distances from the starting line to the middle of the respective colored stripes. It is clearly seen that the localization of cholinesterase practically coincides with the fraction of α-globulins during the electrophoresis of human-blood serum. This is even more clearly evident in Figs. 2 and 3.

The esterase activity is located in other protein fractions in the blood serum of a number of animals. The cholinesterase activity is revealed between the α- and β-globulins, where the coloration characteristic of protein is displayed insignificantly, during the electrophoresis of mouse serum, for example. The characteristic yellow stripe is located between β- and γ-globulins in electrophoresis of guinea-pig blood serum. Thus, the protein which

has the cholinesterase activity moves in the electric field independently of other protein fractions of the serum and may occupy different positions in electrophorograms of blood serum of different animals. However, for the healthy specimens of a species, the character of the distribution of the protein fractions is sufficiently close under standard conditions.

The cited data which characterize the results of a study of electrophoresis in the system of cholinesterase-butyrylcholine, demonstrate only a special case of the application of the principle of countercurrent electrophoresis. In this case the method permitted us to resolve sufficiently definitely the question about the location of the fraction which possesses a biocatalytic activity in the protein spectrum of the blood serum of some animals. The undoubted distinctness and the demonstrability of the results obtained by us permit a recommendation of a wide use of the proposed method both for the work with a series of other enzyme systems and for the characterization of enzyme spectra of biological liquids in the plane of evolutional biochemistry.

SUMMARY

The inclusion of certain indicators (Bromothymol blue and Phenol red) into the composition of buffer mixtures in running paper electrophoresis allows one to follow, during the course of the experiment, the motion of the protein front, and in individual cases, the distribution of the protein fractions as well.

On the basis of the use of indicator-buffer mixtures, we developed a method of countercurrent electrophoresis in a system of enzyme-substrate. With the example of the enzyme system of cholinesterase-butyrylcholine, we showed the applicability of this method for the detection of protein-fraction location on the electrophorogram showing the biocatalytic activity. The site of the meeting of cholinesterase and the substrate is shown in the course of the experiment in the form of a bright-yellow stripe on a blue background.

The fraction which possesses the cholinesterase activity in the protein spectrum obtained by electrophoresis of blood serums of various animals occupies different positions; in other words, the enzymic spectrum of the blood serum of various species of animals differs by a definite specificity.

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THE INFLUENCE OF VARIOUS CONDITIONS ON THE CHEMICAL FIXATION OF PROTEINS ON PAPER

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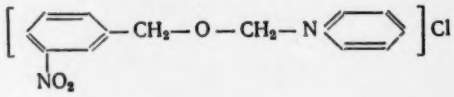
The creation of artificial immunosorbents through fixation of antigens on insoluble carriers is one of the most important problems of immunochemistry [1, 2]. Gurvich [3] proposed for this purpose in 1957 a method of fixation of protein antigens, based on utilization of the halogen compound of the following structure, which reacts energetically with paper:



(I)

On the basis of antigens fixed in this manner, there was developed a new immunochemical method of determination of the amount of antibodies [3], which was used later for the determination of the amount of antitoxins of Cl. oedematiens [4] and antibodies for viruses [5]. An immunosorbent constructed on the same principle has been successfully used for isolation of pure antibodies and for the removal from polyvalent antiserum of one of the species of antibodies (specific "exhaustion" of the antiserum) [6, 7]. Owing to the evident usefulness of such a method of fixation, it was of promise to study the conditions under which the greatest possible amount of protein would be fixed on paper. This is especially necessary for raising the "capacity" of the immunosorbent in preparative isolation of pure antibodies or for "exhaustion" of polyvalent antisera. The knowledge of the most advantageous conditions for the fixation is also very important in the work with viruses, owing to the complexity of preparation of large amounts of purified specific antigens.

TECHNIQUE

The principle of the protein-fixation method described by Gurvich [3] consists of the following. Paper saturated with the solution of the halogen compound shown above was heated to 125°. The residue of the halogen compound is thereby fixed to the paper. A subsequent reduction and diazotization of the nitro group in the residuum of the halogen compound permits one to obtain a salt of the diazonium base which is firmly bound to the paper. In deposition of a protein solution onto the paper which had been treated in this manner, part of the protein molecules are fixed through the diazo group of the residuum of the halogen compound without losing their immunological properties hereby.

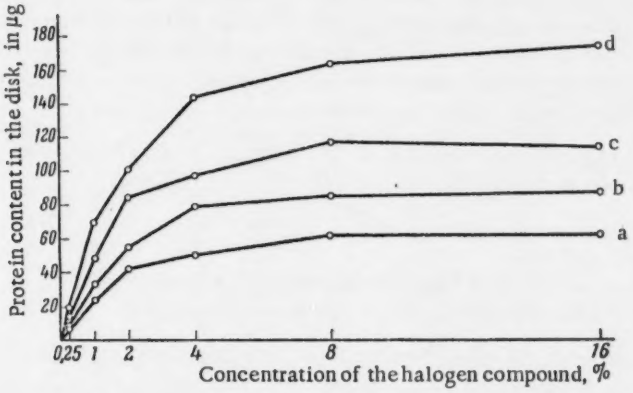
The solution, containing equal amounts of the halogen compound and sodium acetate, was deposited with an automatic pipette, with volume of 41.7 μ liters, onto discs with 2 cm diameter, cut from chromatographic filter paper, Whatman No 1. The disks were heated for 30 minutes at 125° and then were treated as described previously [3] until a free diazo group was obtained in the residuum of the halogen compound attached to the cellulose. Protein solutions were deposited with the same micropipette onto such disks which were marked "experi-

mental." After a 60-minute stay in a moist chamber or in air, the disks were washed for 30 minutes in water and were then placed in a chamber overnight for the final washing and removal of the unbound protein by means of a buffer solution [3]. The protein content in the disks was determined by the amount of acid blue-black dye bound with the disk. For development of the unspecific adsorption, the protein solutions were deposited onto disks which had not been treated with the halogen compound or onto disks with a fastened residue of the halogen compound, the nitro group of which had been reduced to the amino group (diazotization of the latter was not performed). The protein content in such "control" disks indicated the magnitude of the unspecific adsorption and this was subtracted from the amount of fixed protein in the "experimental" disks. The resulting difference indicated the amount of chemically fixed protein on the disk.

RESULTS OF THE EXPERIMENTS

Significance of The Concentration of The Solution of The Halogen Compound Employed.

We studied the effect of the concentration of the solution of the halogen compound, with which the disks were saturated, on the amount of chemically fixed protein. For this, we deposited equal volumes of solutions of human serum albumin of various concentrations onto the disks which had been treated with solutions of the halogen compound in rising concentrations: onto disks of series A – 1.25% albumin solution, of series B – 5%, of series C – 12.5%, and series D – 25% solution. The results of one of such experiments are shown as a graph on the figure, which shows the relationship between the amount of the chemically fixed protein on the disk and the concentration of the halogen compound. It is evident from this figure that the amount of chemically fixed protein rises sharply at first with an increase of the concentration of the halogen compound; a further increase of the concentration of the halogen compound becomes less effective. At 8% concentration, the amount of fixed protein



Relationship between the amount of chemically fixed protein on the disk and the concentration of the halogen compound (I). Onto disks of series A we deposited 1.25% solution of human serum albumin, those of series B – 5% solution, those of series C – 12.5%, and those of series D – 25% solution.

reaches a maximum in all the series of disks studied by us. It is, however, necessary to note that the total amount of free diazo groups on the disk continues to increase with increased concentration of the halogen compound. The increased ability of the diazotized disks to bind β-naphthol with development of an intensely red color indicates the above situation. The question about the causes which prevent the protein molecules from being fastened to the paper through the residue of the halogen compound despite the presence of free diazo groups remains unanswered and requires a further study.

Significance of Concentration of The Protein Solution Deposited.

In order to determine how profitable it may be to increase the concentration of the protein solution which is being deposited, we set up some experiments in which equal volumes of solutions of human serum albumin, differing in concentrations, were placed onto the disks which had been treated with 8% solution of the halogen compound (Table 1).

TABLE 1

Amount of Chemically Fixed Protein Depending of The Concentration of Deposited Protein Solution

The disks were treated with 8% solution of the halogen compound, then the human serum albumin solution in veronal buffer with pH 9.2 was deposited. Time of fixation: 60 minutes at 0° in moist chamber.

Concentration of the deposited solution, %	Amount of protein deposited on each disk, μg	Content of protein, μg		Amount of protein fixed on the disk	
		In "experimental" disk	In "control" disk	μg	%
1,25	521	73	12	61	11,7
5,00	2085	94	12	84	4,0
12,50	5212	130	14	116	2,2
25,00	10 425	186	23	163	1,5

It is evident from Table 1 that the amount of chemically fixed protein rises sharply with increased concentration of the albumin solution being deposited (up to 12. 5 and 25%).

Here, no direct dependence exists between these two magnitudes. The percent of chemically fixed protein relative to that deposited declines with increase of the amount of protein deposited on the disk. The effect of the concentration of the deposited protein solutions on the amount of fixed protein may be evidently explained as follows. In saturating the paper, the protein solution fills the channels whose walls are formed by the cellulose fibers with the residues of the halogen-compound molecules attached to them; only the protein molecules located near to the walls of the channels are able to enter into a reaction with the latter. It is natural that with increased concentration of the protein, a larger number of protein molecules can react with the diazotized residues of the halogen compound attached to the cellulose fibers.

It is also possible to attain an increase of the protein concentration in the paper channels by drying the disks with the protein solutions deposited on them (Table 2).

TABLE 2

The Influence of Drying of Disks, Saturated with The Protein Solution, on The Amount of Chemically Fixed Protein on Them at Various Temperatures

The disks were treated with 1.5% solution of the halogen compound, after which 5% human serum albumin solution in veronal buffer with pH 9.2 was deposited. Time of fixation: 60 min.

Experimental conditions	Temperature of fixation in °C	Protein content, μg		Amount of protein chemically fixed on the disk, μg
		In "experimental" disk	In "control" disk	
Without drying	0	69	11	58
Without drying	20	65	12	53
With drying	20	106	24	82
Without drying	37	69	15	54
With drying	37	129	35	94
Without drying	45	56	20	36
With drying	45	114	45	69

It is evident from Table 2, that, despite the noticeable increase of unspecific adsorption after drying, the amount of chemically fixed protein rises by about 1.5-2 times. A greater part of the protein in the "experimental" disks was fixed through the residuum of the halogen compound, in contrast to the previously described experiments on fixation of protein antigens with simple drying of the paper that had been saturated with protein solutions [8-10].

Even more protein is fixed after repeated deposition of the solution onto the disk, with simultaneous drying. Thus, while in one of the experiments, with a single deposition onto the disk of 41.7 μ liter of 5% human serum albumin solution and subsequent drying there was fixed, through the halogen compound, 117 μg of the protein, 149 μg of the protein was fixed on the disk after a threefold deposition of the same volume of the solution of the protein onto the disk. A similar device had been used previously for increasing the content of virus antigens on disks used in the determination of the amount of antibodies for grippe virus [5].

It follows from the above that for increasing the amount of a chemically fixed protein on paper, it is necessary to increase the protein concentration in the paper channels, by depositing a more concentrated solution onto the disk or by drying the disks which had been saturated with the protein solutions.

Conditions of Fixation.

Effect of Temperature. In the previously cited paper [3], Gurvich ran the fixation of proteins at 0°, which is not always convenient since it hinders a rapid drying of the disks. Therefore we set up some experiments on the study of the effect of different temperatures on protein fixation. It is evident from the data in Table 2 that an elevation of temperature to 37° does not noticeably affect the amount of protein fixed on the disk. The lowering of the amount of fixed protein at 45° may be explained by the rapid destruction of the diazo groups in the residuum of the halogen compound.

TABLE 3

Effect of Time of Fixation on the Amount of Attached Protein

The disks were treated with 2.1% solution of the halogen compound, after which a 5% solution of human serum albumin was deposited in veronal buffer with pH 9.2

Duration of fixation, hours	Amount of protein chemically fixed on the disk, μg
0.5	68
1.0	73
18.0	82
24.0	84

Sucking of Dilute Protein Solutions Through the Disk. At times the necessity may arise to attach a protein to paper, the solution of the protein being available in only a low concentration. In this case one may use the operation of sucking of such a solution through the diazotized disk, using large volumes of the solution. For the sucking through, the disk is laid on a sheet of filter paper over a cut-out hole (1.7 cm in diameter) and a paper wick, dipped into the protein solution, is inserted into the middle of the disk through an appropriately made slit [3]. A comparison of the amounts of chemically fixed protein after the sucking through of 1 ml of 0.2% solution of serum albumin (i.e. in all 2 mg of the protein) and after the deposition directly onto the disk of 41.7 μ liter of the same solution (i.e in all 83.4 μg), showed that in the first case 33 μg of the protein was fixed on the disk through the residuum of the halogen compound, while in the second case 21 μg of the protein was fixed. Thus, the procedure of sucking protein solutions through the diazotized disk may turn out to be useful in some cases.

Significance of the Duration of Fixation. A series of diazotized disks, onto which 5% protein solution had been deposited, was placed in a refrigerator and the fixation was terminated after various time intervals, the disks were washed and the amount of fixed protein in them was determined.

Data in Table 3 show that only a small increase of the amount of chemically fixed protein occurs with increased duration of fixation.

Effect of the Reaction of the Medium of Fixation. Addition to the protein of substances which have a free diazo group occurs evidently better in the alkaline region of pH. Howard and Wild [11] showed that the greatest amount of addition of diazotized p-arsanilic acid to casein may be observed at pH 9.8. However, a necessity may arise to add to paper a protein whose solution has a much less alkaline reaction. In order to check the possibility of fixation under acidic values of pH we deposited onto the diazotized disks some protein solutions which had been prepared in buffers with various pH values.

Data shown in Table 4 indicate that the fixation of protein to the paper may take place over a rather wide range of acidity and even at acidic values of pH.

Fixation of Various Proteins

For clarification of the ability of various proteins to combine with the diazo group of the residuum of the halogen compound, we deposited onto the disks 5% solutions of edestin in 10% sodium chloride solution and 5%

solutions of other proteins being tested in 0.03 N sodium hydroxide solution, considering that the dissolution in alkali would not influence the ability of the proteins to react with diazotized aromatic substances [13]. The protein extracts of organs were obtained by homogenizing the tissue with the same volume of physiological solution and then centrifuging the homogenizate in the cold for 30 minutes at 2500 rpm. For a more precise quantitative accounting of the data we verified the ability of the proteins being tested to bind the acid blue-black dye. It turned out that edestin and vitellin are similar in this respect to the human serum albumin, while glycinin and γ-globulin of horse serum bound the dye less well.

TABLE 4

Effect of pH on Protein Fixation
(the disks were treated with 2% solution of the halogen compound, after which 5% solutions of human serum albumin, prepared with one of the buffers indicated in the table, were deposited on them. Time of fixation was 60 minutes at 0° in a moist chamber. Washing of the disks was done in the corresponding buffers).

Buffer	pH	Protein content, μg		Amount of protein chemically fixed on the disk, μg
		In "experimental" disk	In "control" disk	
Phosphate-citrate	pH 4	91	42	49
»	pH 6	95	21	74
»	pH 7	83	21	62
»	pH 8	86	19	67
Veronal	pH 9,2	65	7	58

The following amounts of the protein were fixed, after the deposition of equal amounts of the various proteins onto disks which had been treated with 2% solution of the halogen compound, with 60 minutes of fixation at 0°:

Name of Protein	Amount of Protein Chemically Fixed on the Disk, μg
Edestin	80
Vitellin	60
Glycinin	50
γ-Globulin of horse serum	85
Casein	60
Water-soluble proteins of mouse liver	103
Water-soluble proteins of mouse brain	77

The above data indicate that the proteins which were tested are fixed in approximately the same degree through the residuum of the halogen compound, despite their different origins and amino acid compositions.

DISCUSSION OF THE RESULTS

It is possible to draw the conclusion from the data shown above that the conditions of the attachment of the protein to the diazotized residuum of the halogen compound bound to the paper do not differ from the conditions of attachment of the protein to the diazonium salts present in solution [11, 12]. An exception is provided by the case, discovered by us, of addition of equal amounts of a protein to the diazotized residuum of the halogen compound fixed on paper, in both alkaline and in weakly acidic medium, while in solution larger amount of the diazo compounds add to the protein at pH 9.8 [11]. The fact that proteins of different origins and amino acid compositions are attached to paper in approximately equal amounts may be explained by the ability of the diazonium salts to react with no fewer than seven amino acids [11].

The above results indicate that for increasing the amount of fixed protein it is necessary, in so far as possible, to increase the concentration of the protein solution being deposited and to use approximately 10% solutions of the

halogen compound. A similar effect is produced by a multiple deposition of the protein with simultaneous drying of the paper; however,in this case one must consider the possibility of denaturation of the protein.

SUMMARY

The conditions of chemical fixation of proteins on paper were studied by the previously described method [3]. It was shown that for obtaining the greatest possible amount of fixed protein it is necessary to deposit a more concentrated protein solution onto paper that had been pretreated with a 10% solution of the halogen compound (I). Considerable amounts of the protein are fixed also after a multiple deposition of the protein solution onto pretreated paper with simulataneous drying of the latter. The nature of the protein is not reflected in its ability to add to the diazo group on the paper.

In conclusion,I express my deep gratitude to A. E. Gurvich for the direction provided during this work.

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ANTIHEMOLYTIC ACTION OF ZINC SALTS

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Zinc salts, as shown in experiments by Smorodintsev and Kapelovich [1], do not cause hemolysis of animal erythrocytes, even at very low concentrations (0.04–0.08%). The mechanism of the antihemolytic action of zinc salts remains insufficiently studied at this time and we failed to find any convincing data in the literature which would explain the reason for the preservation of erythrocytes from hemolysis by solutions of zinc salts which have low concentration and low osmotic pressure.

A study of this problem is the object of the present work.

TECHNIQUE AND RESULTS

The erythrocytes were immediately separated by centrifuging from fresh, defibrinated horse blood.

In the first series of experiments the erythrocytes were mixed with 0.085% solution of zinc sulfate or acetate (in the proportion 0.1 ml of erythrocytes to 3.9 ml of the salt solution).

In the second series of experiments, we added to 0.1 ml of erythrocytes 3.0 ml of physiological solution and 0.9 ml of zinc sulfate solution whose concentration in this mixture ranged from 0.85% to 0.54%.

All the samples were kept at room temperature (16–18°) for 30 minutes, after which the erythrocytes were separated by centrifuging. The supernatant liquid was decanted and the amount of sodium in it was determined (first series of experiments) and that of potassium (first and second series of experiments). Samples of erythrocytes (before and after the action of zinc salts) were subjected to a triple washing with isotonic solution of glucose and the subsequent determination in them of the content of sodium, potassium and zinc. The content of sodium in the erythrocytes and the supernatant liquid was determined by the method of Barensheen and Messiner [2], that of potassium in the erythrocytes according to Breh and Gaebler [3] and that of zinc in erythrocytes, according to the nephelometric method of Gusev, Bitovt and Khor'kov [5].

The amount of potassium in the supernatant liquid was determined by a technique which had been modified by us, according to which the potassium ion was precipitated according to Kramer and Tisdall [4], while the color reaction was run according to Breh and Gaebler. For determination of the color intensity of the solutions, an electrophotocolorimeter was always used.

The hemolysis of the erythrocytes, after the action of zinc salts on them, for the potassium determination was induced by addition of 0.9 ml of 0.01 N sodium hydroxide solution, and for the sodium determination – by 0.9 ml of 0.01 N hydrochloric acid solution. We attained the complete precipitation of the proteins, after the action of coagulating reagents on the hemolyzates and heating in both cases, by keeping the samples in a refrigerator at 2° or 4°.

In the sodium determination, the precipitate of the uranyl – zinc – sodium acetate complex salt was washed with 96%, and not 50%, ethanol, as indicated in the manual [2]. Washing the sample with 50% ethanol causes a partial solution of the sample precipitate which reflects undesirably in the accuracy of the analysis.

The erythrocytes, after the action of zinc salts, were checked for hemolysis in distilled water and blood serum.

The results obtained from the first series of experiments are given in Table 1, from which it is evident that the antihemolytic action of zinc salts is accompanied by adsorption of zinc ions by the erythrocytes and by liberation

from them of sodium and potassium ions, in amounts which are approximately equivalent to the adsorbed zinc ions. Thus, 10.35 mg. equiv. of zinc was adsorbed by the erythrocytes during the action of zinc sulfate and 10, 28 mg equiv. of sodium + potassium was lost, simultaneously, per 100 ml.

The erythrocytes are no longer hemolyzed in water after the action of zinc salts; the passage of hemoglobin from these erythrocytes is noted (with preservation of the sheath) after their immersion into blood serum.

TABLE 1

Change of the Content of Cations in Erythrocytes (E) After Action of Zinc Salts on Them

Erythrocyte samples	Number of samples	Zn-E		K-E		Amt. K liberated into supernatant liq., in mg equiv/100 mg	Na-E		Na liberated into supernatant liq., in mg equiv/100 ml	Hemolysis of erythrocytes in water	Entry of hemoglobin from E into serum
		mg %	mg equiv/100 ml	mg %	mg equiv/100 ml		mg %	mg equiv/100 ml			
Prior to action of zinc salt	15	1,27	0,038	464,0	11,89	0	71,25	3,09	0	+	—
After action of 0,085% $ZnSO_4$ solution	12	339.78	10,39	143,5	3,67	8,48	30,60	1,33	1,80	—	+
» » $Zn(CH_3COO)_2$	3	287,6	8,8	160,6	4,1	7,6	32,6	1,42	1,52	—	+

TABLE 2

Change of the Content of Cations in Erythrocytes (E) After Action of Zinc Salts on Them In the Presence of Sodium Chloride

Erythrocyte samples	Number of samples	Zn-E		K-E		K liberated into supernatant liq., mg equiv/100 ml	Na-E		Na liberated into supernatant liq., mg equiv/100 ml	Hemolysis of E in water	Entry of hemoglobin from E into serum
		mg %	mg equiv/100 ml	mg %	mg equiv/100 ml		mg %	mg equiv/100 ml			
Prior to action of zinc salts	10	1,401	0,043	470,6	12,04	0	63,8	2,77	0	+	—
After action of 0,085% $ZnSO_4$ solution	7	67,07	2,05	438,02	11,2	0,68	35,12	1,52	1,25	+	—
» 0,54% $ZnSO_4$	3	306,0	9,37	155,0	3,96	7,8	30,12	1,30	1,47	—	+

* The average data for the difference between the content of sodium in erythrocytes before and after the action of zinc sulfate are included.

It was noted in a microscopic examination of the erythrocytes which had been subjected to the action of zinc salts (without staining, under a cover glass, magnification 15 × 40 = 600) that they retain the round shape and that their sheath is well defined.

The action of zinc sulfate was tested on erythrocytes in physiological solution, in the second series of experiments.

The resulting data, shown in Table 2, indicate that the action of zinc sulfate on the erythrocytes in the presence of sodium chloride is also connected with the adsorption of zinc ions by erythrocytes and liberation from them of approximately equivalent amounts of sodium and potassium ions, but this process is displayed less clearly

than it is in the action of zinc salts in the absence of sodium chloride. Thus, after the action of 0.85% zinc sulfate solution in physiological solution, the erythrocytes adsorbed five times less zinc ions and liberated a smaller amount of sodium and potassium ions than in the first series of experiments in treatment of the erythrocytes by the same concentration of zinc salts. In this case, the erythrocytes did not change their properties and were hemolyzed after transfer into water. These facts indicate that sodium chloride inhibits the antihemolytic action of zinc sulfate on the erythrocytes.

With increased concentration of zinc sulfate in the samples with physiological solution, its antihemolytic action on the erythrocytes was gradually restored.

Upon reaching the 0.54% concentration of zinc sulfate, the amounts of zinc adsorbed by the erythrocytes and of the sodium and potassium ions liberated by them are approximately the same as after the action of 0.085% zinc sulfate solution in the first series of experiments. The erythrocytes after such treatment again do not hemolyze in water but lose hemoglobin after being placed into blood serum.

In order to determine in which part of the red blood cells the zinc adsorption occurs, we ran some additional experiments in which the separation of hemoglobin from the stroma was done by repeated freezing and thawing of the erythrocytes in isotonic (4.5%) glucose solution. The zinc content was determined separately in the stroma and the hemoglobin.

The resulting average values from five analyses showed that after the treatment of erythrocytes by 0.085% zinc sulfate solution in physiological solution, the main bulk of zinc is adsorbed by the stroma (44.28 mg%), and not by hemoglobin (17.6 mg%) of the erythrocytes. Prior to the zinc sulfate treatment, the content of zinc in the stroma and the hemoglobin of the erythrocytes was 0.22 and 1.32 mg%, respectively. The distribution of zinc ions in the erythrocytes after their treatment with 0.54% zinc sulfate was not studied, since in this case it was impossible to separate the hemoglobin from the stroma.

DISCUSSION OF THE RESULTS

The cited experimental material indicates that absorption of zinc by erythrocytes takes place when they are immersed in solutions of zinc salts.

Since the stroma absorbs considerably more zinc than does the hemoglobin, it is possible to suppose that mainly the stroma proteins are coagulated by the action of the zinc salts and that this hinders the exit of hemoglobin after the subsequent mixing of the erythrocytes with distilled water.

The fact that hemoglobin passes from the erythrocytes after the immersion of them, after the action of zinc salts, into serum, may be regarded as a proof that the permeability of the stroma of the erythrocytes is altered by the action of the zinc salts and that the various compounds of the serum (proteins), which usually do not penetrate the erythrocytes, have in this case an opportunity to pass into the red blood cells and to displace hemoglobin from them.

The presence of sodium chloride in the surrounding medium around the erythrocytes counteracts the antihemolytic action of the zinc salts. Here, the zinc ions are absorbed by erythrocytes in relatively small amounts which, it is possible, cannot bring about the coagulation of the stroma proteins and thus protect the erythrocytes from hemolysis after their immersion into distilled water.

In examining the mechanism of the antihemolytic action of zinc salts it is impossible to fail to note the mutual antagonism of cations of light and heavy metals, which depends on the amount of their salts taking part in the reaction. On one hand, the zinc cations in being adsorbed by the erythrocytes cause the exit of an equivalent amount of sodium and potassium cations from the latter, and on the other hand, the presence of sodium chloride in the surrounding medium counteracts the absorption of zinc ions by erythrocytes and the exit of sodium and potassium ions from them.

SUMMARY

The antihemolytic action of zinc salts is connected with the absorption of a large amount of zinc by the erythrocytes, which is accompanied by the displacement from them of equivalent amounts of sodium and potassium ions. Certain concentrations of sodium chloride protect the erythrocytes from the action of zinc salts. Here, the absorption of zinc by erythrocytes is sharply curtailed, along with the exit of cations of sodium and potassium from them.

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CONCERNING THE ACTIVATOR OF WHEAT-EMBRYO LIPASE

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A large number of papers, which are important not only for the theoretical aspect, but which also have a practical significance, have been devoted to the study of the action of lipase in seeds of the grain plants; with increased lipase activity are connected the biochemical transformations of fat which cause the formation of unpleasant odors and poor gustatory quality of grain products [1–3]. The activity of this enzyme increases, especially with increased moisture in the grain during storage [4]. The greatest lipase activity is possessed by wheat embryos, which fact is evidently connected with the high fat content in them [5, 6].

The present work is devoted to the study of wheat-embryo lipase and to the effect of specific activators isolated from the wheat endosperm.

Defatted flour from wheat germ, of Krasnozerskaya brand, was the source of the enzyme. The wheat embryos were secured from the experimental mill of the All-Union Grain Utilization Research Institute and were carefully separated from impurities. The lipase activity was determined by titration of the resulting fatty acids with 0.1 N sodium hydroxide solution, as described previously [7]; pH optimum 7.2.

It was found during the determination of lipase activity in wheat embryos and endosperm that in the dormant state the lipase activity of the endosperm is below that in the embryos, but is relatively small in either part (2.4 and 1.9 ml of 0.1 N sodium hydroxide solution, respectively). For the experiment we took 1 g of defatted flour from the endosperm or the embryos of wheat. In examination of a mixture of equal amounts (1 g) of wheat embryos and endosperm we found an increase of lipase activity by 2.5–3 times. Evidently, in this case there was displayed the action of a substance which raises lipase activity. For investigation of the nature of this activator we ran a series of experiments.

A water extract was made from the endosperm and the embryos of wheat, and this was added to wheat embryos and endosperm, after which the lipase activity was determined. The precipitates after the water extraction of both parts of the seed were examined in the same manner. Mixtures of the same composition were used as controls, but after a 5-minute boiling, which is needed to destroy the lipase.

1 g of defatted flour from the endosperm (or wheat embryos) was taken for the extraction, 15 ml of distilled water was added, allowed to stand for 30 minutes, centrifuged for 5 minutes at 6000 rpm, and the centrifugate was taken for the experiment. Each experiment was run with no fewer than four repeats.

As is evident from Table 1, the sample in which the precipitate of the endosperm after the water extraction had been added to the wheat embryos has the highest activity (6.7 ml of 0.1 N sodium hydroxide).

Since lipase is soluble in water, the water extract of the wheat embryos and endosperm also gives a sufficient level of activity (5.7 ml of 0.1 N sodium hydroxide solution). Wheat embryos and the aqueous extract of the endosperm, as well as endosperm and the precipitate of wheat embryos after the water extraction, gave values of enzyme activity which were equal to those in the original experiments (pure wheat embryos and pure endosperm). It was established by experiments set up for the refinement of the information about the presence of the activator in a definite part of the grain, that the greatest increase of wheat embryo lipase activity was shown by the endosperm.

An attempt to extract the activator from wheat endosperm was made in the following series of experiments.

TABLE 1

Lipase Activity of Wheat Embryos and Endosperm

Composition of samples	Lipase activity in ml of 0.1 N sodium hydroxide sol. per 1 g of dry wt.
Mixture of embryos and endosperm	6,2
Embryos	2,4
Endosperm	1,9
Endosperm + water extract of embryos	5,7
Endosperm + precipitate of embryos after water extraction	1,9
Embryos + water extract of endosperm	2,8
Embryos + precipitate of endosperm after water extraction	6,7

As is evident from the data in Table 2, the activator is not extracted from the endosperm (remaining in the precipitate) by means of: phosphate buffer with pH 7.2* (0.5 hour, 10 ml of buffer mixture per 1 g), olive oil (10 hours, 4 ml per 1 g), or glycerin (24 hours, 5 ml per 1 g).

In all cases, the centrifugates and the precipitates, obtained after the extraction, were added to the corresponding portions of defatted flour made of wheat embryos.

TABLE 2

Action of Various Extracts of Wheat Endosperm on Lipase Activity of Wheat Embryos

Sample composition	Lipase activity in ml of 0.1 N sodium hydroxide sol. per 1 g of dry wt.
Embryos	3,8
Embryos + extract of endosperm with phosphate buffer mixture, pH 7.2	4,5
Embryos + precipitate of endosperm after buffer extraction	8,1
Embryos + extract of endosperm with olive oil	3,8
Embryos + precipitate of endosperm after oil extraction	8,2
Embryos + glycerin extract of endosperm	4,0
Embryos + precipitate of endosperm after glycerin extraction	8,6
Embryos + precipitate of endosperm after extraction with 10% sodium	3,4

All of the activator passed into solution in treatment of the endosperm with 10% sodium chloride solution in the cold overnight; the solid precipitate no longer possessed any activating ability (Table 2).

In order to determine with which protein fraction of the endosperm, extractable with 10% sodium chloride solution, the lipase activator is bound, we isolated the albumin and the globulin and tested separately their effect on the enzyme activity. For this purpose, the salt extract was centrifuged, filtered through cloth in the cold and dialyzed against distilled water at 4°.

The precipitated globulin was separated by centrifuging from the albumin, by the technique described by Kretovich, Smirnova and Veinova [8], and both proteins were dried lyophilically [9].

The results given in Table 3 indicate that the wheat endosperm albumin and globulin act as activators on wheat-embryo lipase. A mixture of 50 mg of the albumin and 50 mg of the globulin of wheat endosperm has the same action as each of these proteins separately (100 mg). Lipase in its nature is a globulin, but neither the globulin nor the albumin of the endosperm of wheat had any lipase activity.

* Optimum pH of wheat-embryo lipase.

For the determination of the specificity of the activating action of proteins of wheat endosperm, we tested their effect on lipase activity of seeds from other plants (Table 4).

It is evident from Table 4 that wheat-endosperm albumin and globulin raise the lipase activity of seeds of poppy, flax and sunflower by almost twofold.

TABLE 3

Action of Albumin and Globulin of Wheat Endosperm on Lipase Activity of Wheat Embryos

Sample composition	Lipase activity in ml of 0.1 N sodium hydroxide per g of dry weight
Embryos	2,4
Embryos + endosperm albumin (100 mg)	9,5
Wheat embryos + endosperm globulin (100 mg)	10,0
Wheat embryos + mixture of albumin (50 mg) and globulin (50 mg) of endosperm	9,0
Albumin of wheat endosperm	0
Globulin of wheat endosperm	0

It was also of interest to determine whether or not a similar activating action is possessed by the reserve proteins of other plants. For this, we tested the soybean-seed albumin and globulin (Table 5).

The data in Table 5 indicate that the soybean proteins also exert an activating effect on wheat-embryo lipase.

TABLE 4

Action of Albumin and Globulin of Wheat Endosperm on Lipase Activity of Seeds of Poppy, Flax and Sunflower

Sample composition *	Lipase activity in ml of 0.1 N sodium hydroxide sol. per g of dry wt.
Poppy seed	4,7
» » + endosperm albumin of wheat	9,3
Poppy seed + globulin of wheat endosp.	8,8
Flax seed	2,5
» » + albumin of endosperm	5,1
» » + globulin of endosperm	5,8
Sunflower seeds	2,9
» » + albumin of endosperm	7,2
» » + globulin of endosperm	6,5

* In all cases we took for the sample 1 g of defatted flour and 100 mg of the proteins, each.

Thus it is possible to draw a conclusion that the reserve proteins of wheat endosperm and soybean exert an activating influence on wheat-embryo lipase, and those of poppy, flax and sunflower.

For the determination of the purity of the proteins isolated by us, we determined their nitrogen content (with micro Kjeldahl method) (Table 6). The results given in Table 6, in the main, coincide with the appropriate literature data [8, 11].

TABLE 5

Action of Albumin and Globulin of Soybean on Lipase Activity of Wheat Embryos

Sample composition	Lipase activity in ml of 0.1 N sodium hydroxide solution per g of dry weight
Wheat embryos	3.4
" " + soybean albumin	9.4
" " + " globulin	6.9
Soybean globulin	0
" albumin	0

The data on the effect of the amount of the activator on wheat embryo lipase are given in Table 7. These results indicate that with increased amount of albumin, within certain limits (from 25 to 150 mg), the lipase activity rises. After addition of larger amounts of the protein (200 mg), the enzyme activity begins to decline.

Willstätter [10] had determined long ago that albumin exerts an inhibiting action on fat cleavage by the pancreatic lipase in acid medium, but this action is cancelled by the addition of insignificant amounts of calcium chloride or by displacing the reaction to the alkaline side.

TABLE 6

Nitrogen Content in Albumin and Globulin of Embryos of Wheat and Soybean

Protein specimens	Nitrogen content in % on dry weight
Wheat endosperm albumin	12.1
" " globulin	16.2
Soybean albumin	13.3
" globulin	15.1

TABLE 7

Action of the Amount of Wheat-Endosperm Albumin on Lipase Activity of Wheat Embryos

Sample composition	Lipase activity in ml of 0.1 N sodium hydroxide solution per g of dry weight
Wheat embryos + 25 mg of endosperm albumin	4.2
" " + 50 mg of endosperm	4.6
" " + 100 mg " "	8.6
" " + 150 mg " "	9.9
" " + 200 mg " "	8.0

TABLE 8

Action of Wheat-Endosperm Albumin and Calcium Chloride on Wheat-Embryo Lipase

Sample composition	Lipase activity in ml of 0.1 N sodium hydroxide solution per g of dry weight
Wheat embryos	3.5
Wheat embryos + wheat endosperm albumin (50 mg)	7.5
Wheat embryos + $CaCl_2$ *	3.6
Wheat embryos + wheat endosperm albumin (50 mg) + $CaCl_2$ *	7.9

*$CaCl_2$ was added in the amount of 5 ml of 0.1 M solution.

We studied the effect of wheat-endosperm albumin on lipase activity of wheat embryos in the presence and the absence of calcium chloride (Table 8).

It is evident from Table 8 that calcium chloride does not exert any action on lipase activity of wheat embryos, in contrast to the pancreatic lipase, nor does it affect the activating effect of the endosperm albumin on this enzyme.

SUMMARY

Wheat endosperm and soybean seeds contain substances which activate the wheat-embryo lipase. The activator is not extracted by water, phosphate buffer, olive oil or glycerin, but is extracted by 10% sodium chloride solution.

The albumins and globulins, isolated from the wheat endosperm and soybean seeds, activated the lipase of embryos of wheat, seeds of poppy, flax and sunflower. Thus, the reserve proteins of wheat endosperm and soybean exert an activating action on a series of plant lipases.

I consider it my duty to express my gratitude to Professor A. A. Prokof'ev for his valuable advice during the course of this work.

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OXIDATION OF ASCORBIC ACID BY OXIDASE SYSTEMS OF WHEAT FLOUR

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At the present time ascorbic acid is beginning to acquire a wide distribution in a number of countries as an effective improving agent in baking of bread, replacing potassium bromate, owing to the undoubted advantages possessed by the former.

The improving effect of ascorbic acid in baking was first noted by Jorgensen [1] who, however, failed to give any explanation of this fact. Melville and Shattock [2] supposed that ascorbic acid becomes a bread improver only after its transformation into the dehydro form under the influence of the enzyme present in the flour and is provisionally taken to be ascorbinoxidase. Studies made by Proskuryakov and Pavlinova [3] showed that an enzyme system indeed exists in wheat flour, which causes oxidation of added ascorbic acid and resembles ascorbinoxidase in a number of its properties.

The final solution of the problem of the nature of the enzymic oxidation of ascorbic acid in wheat flour is made difficult by the presence of several enzyme systems in plant objects which are capable of producing this oxidation.

Szent-Gyorgyi [4] long ago found the enzyme which causes the direct oxidation of ascorbic acid and which was later named ascorbinoxidase or ascorbinase. The same author [5], followed by Tauber [6], Engel'gardt and Bukin [7] and Rubin and Chetverikova [8], showed the possibility of oxidation of ascorbic acid in the presence of hydrogen peroxide by means of plant-juice polyphenols. Oxidation of ascorbic acid by polyphenolase in the presence of various polyphenols was discovered in the work of Mikhlin [9] Engel'gardt and Bukin [7], and Bokuchava, Shubert and Popov [10], while Keilin and Mann [11], Keilin and Hartree [12] and Rubin and Chetverikova [8] showed the possibility of oxidation of ascorbic acid by cytochrome oxidase through cytochrome C. Quite recently, Maltha [13] discovered the ability for indirect oxidation of ascorbic acid by the glucosoxidase of the mold fungi.

Thus, the problem of the nature of the enzyme system, present in the grain products, which causes the oxidation of ascorbic acid, is considerably complicated by the possible presence in it of at least four enzymes.

The aim of this work was the clarification of the actual nature of the enzymic oxidation of ascorbic acid by aqueous extracts and suspensions of wheat flour. It is necessary to remark, however, that after the inactivation of the enzymes by heating the aqueous extracts and suspensions of the flour, the oxidation of the added ascorbic acid still takes place on a rather considerable scale through the as-yet-further-undetermined thermostable factors.

EXPERIMENTAL

Preparations, Setting up of Experiments and Analytical Methods. The preparation of ascorbinoxidase with high activity was made from the bark of Cucurbita pepo, according to the method of Dawson and Magee [14]; the peroxidase preparation was obtained from horse-radish roots according to Kenten and Mann [15].

To aqueous extracts of wheat flour or to aqueous suspensions of the flour, we added a definite amount of a freshly prepared ascorbic acid solution and after allowing this to stand at 30–34° for various periods of time, a solution of HPO_3 was added to the mixture up to its final concentration of 2%. The residual ascorbic acid was determined by titration according to Fujita and Ebichara [16]; the oxidized acid was determined by the difference be-

tween its original amount and that remaining after the experiment. The controls were set up in the same manner, but the HPO_3 was added prior to the addition of ascorbic acid. The water for the experiments was distilled twice in glass apparatus.

RESULTS OF THE EXPERIMENTS

Oxidation of Ascorbic Acid in Flour Suspensions and Extracts

First of all an attempt was made to determine whether or not any difference exists in the intensity of summary oxidation of ascorbic acid after its introduction into aqueous suspensions and extracts of flour made from nine different brands of wheat, and also to determine the fraction of oxidation caused by the thermostable factor.

TABLE 1

Amount of Ascorbic Acid Oxidized Enzymically by Flour Suspensions From Various Brands of Wheat

Brand of wheat	Ascorbic acid	
	mg/ 0.25 g of flour	% of total amount
Liutestsens 230 (A)	0.075	49.4
Albidum 43	0.081	62.4
Omsk 2078	0.069	58.0
Odessa 3	0.052	42.6
Liutestsens 230 (C)	0.066	42.0
Belotserkovskaya 198	0.071	44.1
Pimenka	0.067	46.5
Ukrainka	0.060	57.7
Karaganda 92	0.074	60.0

To 2.5 g samples of the flour we added 25 ml of 10 mg% solution of ascorbic acid, each and the mixtures were placed in flasks at 34° for 4 hours and after each 30 minutes 25 ml of 4% HPO_3 was added to each flask; after centrifuging, the titration was run. It was shown that with this total oxidation of ascorbic acid by suspensions of first-class flour obtained from nine different wheat brands, noticeable differences existed (from 46.8 to 66.1%) while $^2/_3$ of the total amount of the acid was oxidized within the first two hours.

Similar samples of the flour were subjected to a half-hour heating with live steam and the experiments with these were run in a similar way. It turned out that the ascorbic acid was also oxidized in these cases.

Taking the amount of summary oxidation in the 4-hour experiments as 100%, the amount of enzymically oxidized ascorbic acid amounted to from 42 to 62.4% (Table 1).

Thus it is possible to assume that under the conditions of these experiments about half of the total added ascorbic acid was oxidized by the oxidase systems of the flour, and the other half by the thermostable factor.

Similar experiments were run with aqueous extracts of the same flour samples, with the extracts having been prepared by one hour's standing with water (1:2) with subsequent centrifuging.

It turned out that the aqueous extracts oxidized the ascorbic acid considerably more weakly than did the flour suspensions. The fraction of the purely enzymic oxidation by the extracts was 1.5–2 times smaller than with the flour suspensions. This made us suppose that part of the system, which causes specifically the enzymic oxidation of ascorbic acid, is bound most firmly with the flour particles.

Oxidation of Ascorbic Acid by Preparations of Ascorbinoxidase and Peroxidase

In the first place it was necessary to determine which of the indicated enzyme systems are present in the extracts and the suspensions from the flour.

No polyphenolase and cytochrome oxidase activity was found in the flour suspensions, after tests by the manometric method in the Warburg apparatus, which fact agreed with the data from Waygood [17], while at the same time even the aqueous extracts from the flour contained a very active peroxidase as determined by the method of Sumner and Gjessing [18].

The flour suspension noticeably absorbed oxygen in the presence of ascorbic acid in the Warburg apparatus, which, in the absence of polyphenoloxidase and cytochrome oxidase, was an evident proof of the presence of ascorbinoxidase in the flour.

On this basis, we set up 4-hour model experiments on the action of the preparations we had made – ascorbinoxidase and peroxidase – on ascorbic acid, both separately and jointly with the flour extracts and suspensions.

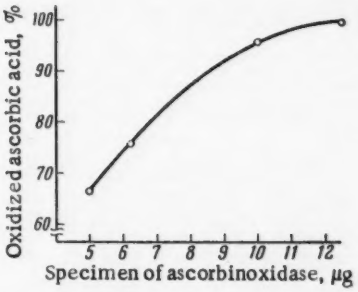
It is shown in Table 2, that the addition of ascorbinoxidase preparation to the flour extract sharply raised the oxidation of ascorbic acid taken in an appreciable amount.

Similar results were obtained also in experiments with the flour suspensions, but the intensity of oxidation was considerably higher in this case.

TABLE 2

Oxidation of Ascorbic Acid by a Preparation of Ascorbinoxidase, Separately and Jointly with a Flour Extract. 5 ml of 25 mg% ascorbic acid solution was added to each sample

Extract from flour (1:2), ml	Specimen of ascorbinoxidase, μg	Water, ml	Oxidized ascorbic Acid	
			in mg	%
5	—	10	0.169	13.5
—	5	10	0.677	54.0
5	5	5	0.803	64.0



Effect of different concentrations of the preparation of ascorbinoxidase on oxidation of ascorbic acid.

The rate of oxidation of ascorbic acid increased correspondingly with increase of the amount of the ascorbinoxidase preparation (see figure).

Similar 4-hour model experiments were run also with the peroxidase preparation with the simultaneous presence of hydrogen peroxide and pyrogallol. In the control we determined the individual oxidation of ascorbic acid by hydrogen peroxide, pyrogallol, flour suspension and extract, and the enzymic preparation. It is evident from the data in Table 3, that the peroxidase preparation actually oxidized ascorbic acid rather intensely in the simultaneous presence of hydrogen peroxide and pyrogallol (by 67.8%), while in the control run, the preparation and hydrogen peroxide alone oxidized only 23.2% (15.8 + 7.4%). With the joint action of the preparation and the flour extract on the substrate, the experimental oxidation of ascorbic acid was 38.6%, and that in the control 41% (17.8 + 15.8 + 7.4%). With the use of the flour suspension, the control amounted to 91.4% (68.2 + 15.8 + 7.4%), while the experiment gave 87.1% oxidation.

TABLE 3

Oxidation of Ascorbic Acid by Peroxidase Preparation, Individually and Jointly with Flour Extract and Suspension.
To each sample we added 5 ml of 25 mg% solution of ascorbic acid

Extract from flour (1:2) ml	Flour, g	H_2O_2, 0.01% solution, ml	Peroxide preparation, μg	Water, ml	Pyrogallol, mg	Oxidized ascorbic acid	
						in mg	%
4,5	—	—	—	5,5	—	0,2184	17,8
—	2,5	—	—	10	—	0,8390	68,2
—	—	0,5	—	9,5	—	0,1946	15,8
—	—	—	200	10	—	0,0912	7,4
—	—	—	—	10	10	0	0
—	—	0,5	200	9,5	10	0,8320	67,8
4,5	—	0,5	200	—	10	0,4740	38,6
—	2,5	0,5	200	9,5	10	1,0700	87,1

Consequently, the peroxidase preparation apparently lost the ability to oxidize ascorbic acid in combination with the flour extracts and suspensions, which most probably occurred through the rapid decomposition of hydrogen peroxide by the quite-active flour catalase.

Later we set up experiments for the determination of the question as to whether or not dehydroascorbic acid is the sole oxidation product of ascorbic acid from the action of wheat-flour ascorbinoxidase.

For this purpose, the filtrates after the 4-hour oxidation were treated with hydrogen sulfide and, after its complete displacement with carbon dioxide, the amount of ascorbic acid was determined. It turned out with the flour extracts that all the added ascorbic acid passed into the dehydro form which was completely reduced. In case of the flour suspension, however, it was found that with oxidation of about $^2/_3$ of ascorbic acid to the dehydro form, about $^1/_3$ was oxidized to, evidently, more profound decomposition products.

Effect of Inhibitors on Oxidation of Ascorbic Acid by Flour Suspensions and on the Physical Properties of Dough

For a final proof of the existence of ascorbinoxidase in the wheat flour and for the establishment of its role in the total oxidation, we set up some special experiments using certain inhibitors: KCN and diethyldithiocarbamate sodium salt, in final concentrations, respectively, of 0.01 M and 0.005%. It is possible to suppose that the inhibiting effect after the addition of potassium cyanide would be greater or at least equal to that produced in the presence of sodium diethyldithiocarbamate. However, it is evident from the data in Table 4 that the greater inhibiting action was produced by sodium diethyldithiocarbamate. Rubin and Chetverikova [8] had noted that under certain conditions potassium cyanide not only failed to inhibit, but actually considerably activated the process of oxidation of ascorbic acid by plant suspensions.

TABLE 4

Action of KCN and Sodium Diethyldithiocarbamate on Oxidation of Ascorbic Acid by Flour Suspensions.
Amount of oxidized ascorbic acid given in mg

Duration of experiment, hours	Without inhibitor, mg	With KCN		With sodium diethyldithiocarbamate	
		mg	inhibition, %	mg	inhibition, %
0.5	0.288	0.192	33.4	0.156	45.8
1.0	0.424	0.200	38.7	0.220	48.0

Therefore, the action of cyanide as an inhibitor of metal-carrying oxidase, cannot be considered as being absolute and one should approach the resulting data with caution. However, the inhibition of the oxidation process of ascorbic acid produced by sodium diethyldithiocarbamate, which acts specifically only on ascorbinoxidase and polyphenoloxidase [14], is the direct proof of the presence in the flour of, specifically, ascorbinoxidase (insofar as polyphenoloxidase is absent in it) as the only enzyme system of the wheat flour that produces oxidation of ascorbic acid.

TABLE 5

Effect on the Physical Properties of Dough ("W") of Ascorbic Acid Jointly With the Enzymic Preparation and its Inhibitor

Conditions of experiment	"W"
Control dough	158,5
Dough + 12,5 mg of ascorbic acid	204,9
» + 12,5 » » » + 20 mg of enzyme preparation	201,8
» + 12,5 » » » + 20 » of inhibitor	166,7
» + 12,5 » » » + 50 » »	150,8
» + 12,5 » » » + 100 » »	138,5

Finally, we made an attempt to apply the conclusions from the previous experiments to the problem of the mechanism of action of ascorbic acid as an improver of the physical properties of dough, using as a basis Zaglodina's data [19].

Insofar as the bettering action of ascorbic acid is connected to its transition into the dehydro form, which is a direct oxidizing agent, one may assume that the addition of a preparation of ascorbinoxidase to the flour should enhance the effect of ascorbic acid on the betterment of the physical properties of the dough and, conversely, addition of an inhibitor of ascorbinoxidase should to some degree lower the quality. For checking this supposition, we ran some tests of the properties of dough, using the Chopin alveograph, with addition of the enzyme preparation and its inhibitor, while "W", as the measure of the "strength" of the flour, was taken for the principal index after a 20-minute layover of the dough (Table 5).

Addition of the ascorbinoxidase preparation to the dough, which contained ascorbic acid in the amount of 0.005% based on the weight of the flour, failed to give a notable improvement of its properties and "W" remained almost without change. At the same time, the effect of sodium diethyldithiocarbamate was detected quite clearly, especially with increased doses of it.

The absence of the effect after the addition of ascorbinoxidase preparation on the "W" index may be explained thus: all the added amount of ascorbic acid was rapidly oxidized by its own ascorbinoxidase.

To confirm this, we ran experiments on oxidation of ascorbic acid over different time intervals, not in water suspensions of flour, but in stiff, kneaded dough, with the dose of ascorbic acid corresponding to the amount of it used for mixing the dough in the alveograph (0.25 mg per 5 g of flour). The results showed that after even 20-minute layover of the dough, the added ascorbic acid was totally oxidized. Consequently, with such insignificant doses, theascorbic acid is easily oxidized even without the addition of ascorbinoxidase preparation.

SUMMARY

It was shown in a comparison of the summary oxidizability of ascorbic acid by water extracts and suspensions of flour from nine different brands of wheat, that under these conditions of the experiments all samples showed a considerable ability to oxidize the former (up to 47-66% of the amount taken).

The most important, and evidently the only, enzyme system of wheat flour which participates in the oxidation of ascorbic acid, is ascorbinoxidase, which has a rather high activity. The action of ascorbinoxidase present in wheat flour may be severely depressed by the introduction of a specific inhibitor – sodium diethyldithiocarbamate.

The bettering action of ascorbic acid on the physical properties of dough is caused by its rapid transition to the dehydro form, which is an energetic oxidizing agent.

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EFFECT OF ANSERINE ON OXIDATIVE PHOSPHORYLATION IN MUSCLE TISSUE

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The problem of the role of the dipeptides – carnosine and anserine – in the processes of metabolism of muscle tissue remains open to this day. As was shown previously by us [1, 2], addition of anserine raises the amount of phosphocreatine formed in the incubation of muscle macerate in the presence of creatine as the final phosphate acceptor. In experiments with addition of carnosine, the increase of formation of phosphocreatine was noticeable, but considerably smaller than after the addition of anserine. On the basis of these data a supposition was expressed that anserine has a specific effect on respiratory phosphorylation of muscle tissue, which differs from the action of carnosine [2].

Sherstnev [3], in studying the effect of carnosine on the processes of phosphorylation of muscle tissue, drew the conclusion that the effect of carnosine on respiration and phosphorylation is caused only by the buffer properties of carnosine.

In our earlier studies, the pH determinations were made with the colorimetric method, using the universal indicator. Considering that with this method the accuracy of pH determination was insufficient and that the pH difference could unquestionably affect the intensity of respiration and phosphorylation, we returned again to the experiments on the study of the effect of anserine on oxidative phosphorylation.

EXPERIMENTAL AND DISCUSSION

The pH determination in the present work was done by the potentiometric method with glass electrdes in the experimental samples and before and after the incubation. The experiments were run with macerated pigeon breast muscle in phosphate buffer solution. For phosphate acceptor we added creatine in the amount of 10–12 mg per 3-ml-volume sample. Samples of the tissue weighed 200–300 mg, the amount of anserine was 25–30 mg. The setting up of the experiments was as follows.

Into the Warburg vessels or into flasks (when the experiments were run without account of the respiration), containing the buffer solution with all the addends*, we placed tissue samples and passed oxygen into these over 3–5 minutes. Then, one part of the samples was incubated, while in the other part we immediately ran a pH determination and selected samples for the determination of labile phosphorus in the protein-free filtrate, after the precipitation of inorganic phosphate with magnesia mixture [4].** Phosphocreatine was determined in a separate portion by the Alekseeva method [5]. These samples, prior to incubation, were considered to be the starting samles. The incubation was run at 26° with constant shaking over 120 minutes *** The determination of phospho-

* Composition of the buffer solution: phosphate – 100 μ moles; KCl – 3.5 μ moles; $MgSO_4$ – 1.3 μ moles; $NaHCO_3$ – 27 μ moles; NaCl – 300 μ moles per 3 ml; pH 8.4–8.5 for samples without anserine; 7.3–7.5 for samples with anserine.

** Phosphocreatine and other unstable (in acid medium) phosphorus compounds, which behave like phosphocreatine, entered the composition of the labile phosphates.

*** Duration of incubation in some cases was 30, 60 and 90 minutes.

creatine and labile phosphate before and after the incubation was run as follows. After the pH determination, the samples were filtered through cheesecloth layer into test tubes immersed in snow. The filtrate was immediately picked up in a pipette and poured: 1) into an equal volume of 1.4% ammonium molybdate solution prepared in 2 N sulfuric acid (for determination of phosphocreatine) and 2) into an equal volume of 5% trichloroacetic acid solution (for determination of labile phosphate).

Considering that anserine has a buffer activity and that for this reason the addition of the muscle macerate to samples with anserine lowers the pH level less drastically, the initial phosphate solution for the samples incubated with anserine had a lower pH level than did the samples incubated without it. This permitted us to maintain the pH of the test samples practically the same toward the end of incubation (Table 1).

TABLE 1

Change of pH of Solutions Being Tested in the Course of Incubation

Date	Sample composition	pH			
		Initial buffer solution	Sample prior to incubation	Sample after 60 min incubation	Sample after 120 min incubation
15. V 58	No anserine	8,51	7,50	7,15	7,08
	With anserine	7,41	7,32	7,19	7,08
24. V »	No anserine	8,48	7,41	6,92	6,96
	With anserine	7,40	7,19	6,99	6,98
7. VI »	No anserine	8,47	7,66	7,10	7,04
	With anserine	7,24	7,20	7,08	6,96
30. VI »	No anserine	8,46	7,40	—	7,25
	With anserine	7,35	7,13	—	7,01

TABLE 2

Content of Labile Phosphate and Phosphocreatine in μg of P in Test Sample With 3 ml Volume and pH of Starting Samples. Samples prior to incubation

Date	Tissue sample, mg	pH		Labile phosphate		Phosphocreatine	
		no anserine	with anserine	no anserine	with anserine	no anserine	with anserine
15. V 58	300	7,50	7,32	192	135	163	108
24. V »	400	7,40	7,19	302	270	290	245
7. VI »	200	7,66	7,20	113	65	165	67
30. VI »	200	7,40	7,13	120	93	78	64

In the original samples (prior to incubation) with anserine, the pH of the solution was, as a rule, always lower than in samples without anserine. The higher value of the pH in the original solutions is explained by the high content of labile phosphate and phosphocreatine in the samples prior to incubation without anserine. Actually, these samples are samples in which an approximately 10-minute incubation had taken place (duration of oxygen saturation and pH determination (Table 2).

It was shown in the preliminary experiments that the accumulation of labile phosphate is practically completed in 45–60 minutes of incubation, after which the content of labile phosphate in the samples being incubated remains practically constant over two hours or even drops slightly (Fig. 1).

The results of three incubation experiments are shown in Fig. 1 for 200 mg of the tissue. In the experiment done on 6 IV 57 (3), α-ketoglutaric acid (2 mg per sample) was added as the respiration substrate.

The amount of resulting labile phosphate also depends on the amount of added creatine. The amount of determinable labile phosphate increases in the test samples with increase of creatine concentration, while respiration remains practically constant (Fig. 2).

The formation of labile phosphate is determined by the amount of ATP and creatine. The content of ATP in the samples being incubated depends on the intensity of its formation and decomposition, which is determined

by the activity of the individual enzyme systems. The activity of the enzymes, in turn, is determined by the concentration of the tissue. Specifically, the activity of ATP-ase rises sharply with increased amount of the tissue, or, more correctly, its concentration. Experiments set up by Razumovskaya [6] on the determination of ATP-ase activity, showed that the enzyme activity rises sharply with increased amount of the added tissue. With large tissue samples, the sharp rise of ATP-ase may lead to a relatively smaller accumulation of labile phosphate. Actually, the experiments set up with various sizes of tissue samples showed that only with small amounts of the tissue does one observe a proportionality between the amount of the tissue and the amount of labile phosphate formed. With large tissue samples, the formation of labile phosphate is lowered sharply (Fig. 3).

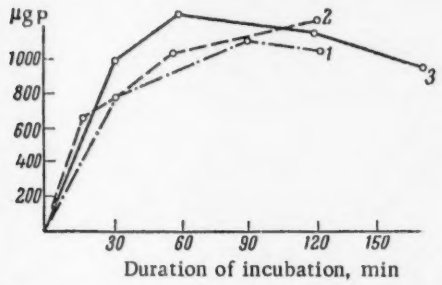

Fig. 1. Amount of labile phosphate determinable at various periods of incubation.

Thus, in characterizing the intensity of oxidative phosphorylation by the amount of determinable labile phosphate, one must strictly account for the amount of tissue used and for the amount of added creatine. The maximum which is observed for the labile phosphate content with prolonged incubation of muscle tissue in the presence of creatine, is determined by the establishment of equilibrium of the rates of reactions which cause the formation and the decomposition of ATP and does not indicate a disturbance of the phosphorylation process as yet.

In studying the effect of anserine on the process of oxidative phosphorylation of muscle tissue, we took such amounts of the tissue (200–300 mg per 3 ml) that the sharp rise of ATP-ase activity was not observed; formation of labile phosphate was proportional to the amount of added tissue. The resulting experiments on the determination of the amount of consumed oxygen showed that the addition of anserine practically does not change the intensity of respiration, which remains constant during the 2-hour incubation. The amount of labile phosphate, however, was considerably greater in the samples with anserine. Especially clear was the difference between the samples after a two-hour incubation. As a rule, a more or less clearly defined decrease of the amount of labile phosphate was observed by this time in samples without added anserine. In samples with anserine such a decrease was considerably smaller, if it was observed at all (Table 3, Fig. 4).

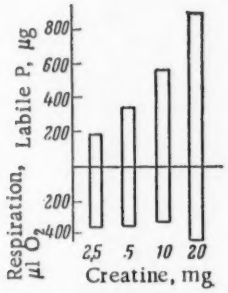

Fig. 2

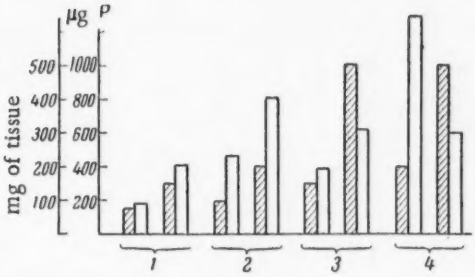

Fig. 3

Fig. 2. Effect of the amount of added creatine on respiration and formation of labile phosphate in test samples.

Fig. 3. Dependence of formation of labile phosphate on amount of tissue. Shaded bar – tissue; unshaded bar – labile P. Dates of experiments: 1) 11 III 58; 2) 13 III 57; 3) 3 III 57; 4) 9 V 56.

The amount of labile phosphate was always somewhat greater than the amount of determinable phosphocreatine, but this difference was practically the same for both the controls and the samples with anserine.

Accurate determinations of pH were run in the present study during the entire incubation period. We succeeded to create conditions, in the preparation of the buffer solutions with various pH values, under which the pH values were practically the same after 60 and 120 minutes of incubation in samples with anserine and without it (Table 1). Despite this, the addition of anserine always led to a large accumulation of labile phosphate after 60-and, especially, 120-minute incubation (Table 3 and Fig. 4). With 30-minute incubation the content of labile phosphate differed but little in samples with anserine or without the latter.

TABLE 3

Effect of Anserine on Respiration and Formation of Labile Phosphate and Phosphocreatine

Date	Amt. of tissue, mg	Duration of incubation, min	pH		Absorption of O_2, µl		Labile P, µg		Prosphocreatine, µg	
			without anserine	with anserine	without anserine	with anserine	without anserine	with anserine	without anserine	with anserine
15. V 58	300	60	7,15	7,19	—	—	756	840	729	769
» » »	»	120	7,08	7,08	—	—	414	627	379	640
24. V »	400	60	6,92	6,99	—	—	1194	1284	1030	1115
» » »	»	120	6,96	6,98	—	—	720	954	637	823
7. VI »	200	60	7,10	7,08	—	—	667	825	507	744
» » »	»	120	7,04	7,96	—	—	571	818	411	695
30. VI »	200	120	7,25	7,01	893	1054	265	753	261	710
4. VII »	200	60	7,06	7,00	493	459	569	796	557	714
» » »	»	120	7,10	7,96	910	870	569	990	525	868

As a result of the study of the effect of anserine on oxidative phosphorylation in muscle tissue, we cannot agree with the conclusion made by Sherstnev [3] that the effect of this dipeptide on oxidative phosphorylation is limited by its buffer action. This conclusion of the author is condidered to be erroneous by us since the organization of the experiment that he did was unsuitable for the judgment of the intensity of oxidative phosphorylation.

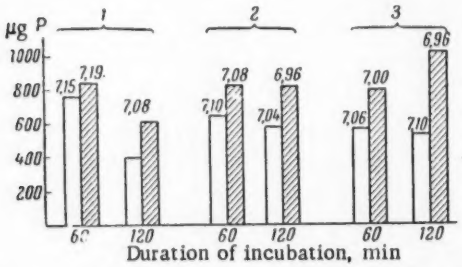

Fig. 4. Effect of anserine on formation of labile phosphate.

Unshaded bar — control; shaded bar — with anserine. Dates of experiments: 1) 15 V 58; 2) 7 VI 58; 3) 4 VII 58; numbers above bars — pH of the experiments.

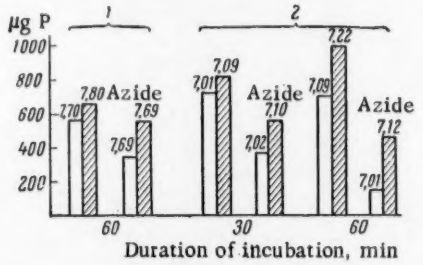

Fig. 5. Effect of anserine on formation of labile phosphate in the presence of azide. Azide concentration: 1) $8.5 \cdot 10^{-4}$ M; 2) $7.75 \cdot 10^{-4}$ M. Dates of experiments: 1) 5 VII 57; 2) 25 III 58. Numbers above bars indicate pH of the experiment.

Actually, addition of large amounts of sodium fluoride (0.025–0.50 M) causes an inhibition of succinodehydrogenase [7] and brings about a sharp drop in the oxidation processes. In addition, neither glucose nor hexose-6-phosphate may undergo a direct oxidation in the skeletal musculature since the muscle tissue does not contain the appropriate enzymes. In Sherstnev's experiments pyruvic acid cannot form from the added glucose and serve as a respiration substrate since the added sodium fluoride excludes any enolase activity. Finally, it is well known that experiments on characterization on oxidative phosphorylation based on the decrease of mineral phosphate during incubation cannot be run without adding systems which accept the mineral phosphate. In Sherstnev's experi-

ments, which we are discussing, this condition was not met since neither creatine nor hexokinase was added to the experimental samples. Thus, the glucose, under this setup of the experiments, could not serve as a substrate for oxidation nor as an acceptor of phosphate and the purpose for its addition is not clear. The drop in the mineral phosphate in Sherstnev's experiments cannot, by any means, be characteristic of the intensity of oxidative phosphorylation. Finally, in this paper of Sherstnev there are given some experimental data obtained after the addition of carnosine to the experimental samples. However, we had stressed the favorable effect of anserine on oxidative phosphorylation.

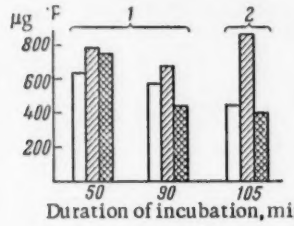

Fig. 6. Effect of anserine and histidine on formation of labile phosphate.

Unshaded bar – control; inclined shading – anserine; cross-hatching – histidine. Dates of experiments: 1) 9 IV 58; 2) 2 IV 58.

Returning to the description of the results obtained by us, it should be noted that in experiments with the azide at concentrations of $7.5 \cdot 10^{-4}$ M, sufficient for repression of phosphorylation without a noticeable change in respiration, an addition of anserine brings about again a large, in comparison with the control, accumulation of the labile phosphate. In experiments with the azide, at all durations of incubation, the difference between the amount of labile phosphate in samples with anserine and those in the control was always relatively larger than in the experiments without the azide (Fig. 5).

It is possible to conclude, on the basis of the data obtained by us, that anserine preserves for a longer period of time the normal course of the oxidative phosphorylation process. A disturbance of the oxidative phosphorylation process leads to a deformation of the cell structure [8] which in turn leads to a rapid disturbance of the enzymic processes in general and, first of all, of the processes of oxidative metabolism. In work done with mitochondria [9] obtained from the pigeon breast muscle, it was shown that the addition of anserine to the "aging" mitochondria protects them from swelling and maintains the process of oxidative phosphorylation in them at the level which is characteristic of freshly obtained mitochondria. Without such addition of anserine, the mitochondria swell rapidly and the process of phosphorylation in them is noticeably disturbed.

We did not observe an effect similar to that with anserine during incubation of muscle macerate with histidine, just as in the experiments with the mitochondria: addition of histidine failed to lead to a large accumulation of labile phosphate after a prolonged incubation (Fig. 6).

Thus, the resulting data indicate a special specific role of anserine in the processes of oxidative phosphorylation of the muscle tissue, which consists of its protective action in respect to energy-rich phosphorus compounds. The preservation of the macroenergetic compounds at a certain level preserves the cell structure and protects the enzymic processes from disturbances.

SUMMARY

The effect of anserine on oxidative phosphorylation in muscle tissue was studied in the presence of creatine as the phosphate acceptor. It was established that the addition of anserine, with prolonged incubation, maintains the formation of macroenergetic phosphorus compounds at a higher level. It is impossible to explain this effect by a buffer action of anserine, since the pH values in samples with anserine were the same as without the latter. One may rather speak of the protective action of anserine on the macroenergetic phosphorus compounds.

Histidine does not cause an effect similar to that of anserine.

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** Russian translation

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THE EFFECT OF SORBITOL ON THE COURSE OF CHOLINE DEFICIENCY

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The investigation of Morgan and Yudkin [1] and also of Griffith, Morgan, and Yudkin [2] showed that the development of thiamin avitaminosis in rats was greatly hindered if the artificial diet of the rats included sorbitol instead of glucose. This was related to prolongation of life, increase in weight, and disturbance in function of the nervous system. The experiments showed that if all vitamins of the B group were excluded from the experimental diet except choline and inositol (with the initial carbohydrate being sucrose), then in this form of combined vitamin deficiency, sorbitol also showed the ability to cut down the requirements of the organism for some of the B vitamins. Control experiments with exclusion of choline or inositol from the diet gave the same results. Further studies [3] showed the ability of D-sorbitol to improve the assimilation of vitamin B_{12} by the organism.

In the control experiments of these authors with the inclusion of sorbitol in the diet and exclusion of choline, the animals received 20% casein, and the indicator of the results obtained was the intensity of growth (gain in weight). However, the presence in the diet of 20% casein was in itself sufficient to guarantee active methyl groups (from methionine) to the rats and thus to avoid the appearance of a choline deficiency; also, the gain in weight is not always a sufficient sign of absence of a choline deficiency. Specific evidence of a choline deficiency which can serve as evidence of such a lack in the organism is the characteristic change in the kidney and fatty infiltration of the liver; these changes occur even on the first day of a choline deficiency. The question of the possibility of intensifying the endogenous formation of choline by including definite substances in the diet assumes greater importance because choline deficiency leads, under experimental conditions, to such serious pathological processes as hypertrophy, atherosclerotic lesions of the major and coronary vessels, cirrhosis of the liver, and malignant tumors [4, 5]. Therefore, it seemed a worthwhile possibility to prevent the development of such serious and complex pathological processes, even if only in model experiments, by changing the character of only one type of the intestinal flora by including specific substances in the diet. It should be mentioned that even in 1947 Baxter [6] actually showed that the nature of the carbohydrate part of the dietary ration could affect the demand of the organism for choline.

In these experiments we included corn starch instead of sucrose in the rat diet with the express purpose of hindering the development of a choline deficiency.

EXPERIMENTAL

In the experiments we used male rats weighing 40–50 g.

The experiments were so carried out that the animals had a diet deficient in methionine and choline; one groups received sucrose as the carbohydrate and the other received sorbitol. Evaluation of the effect of each diet was made by the length of life, increase in weight, and development of such basic effects of choline deficiency as change in kidneys and accumulation of fat in the liver. Three groups of rats were used in the experiments.

The first (control) group of rats (16 animals) received a basic diet which contained a slight amount of methionine and was freed from choline by extraction with alcohol. As the basic diet we used the nutritive mixture of Copeland and Salmon [7]. The chief source of protein in this diet was peanuts, which are very low in methionine. We introduced into the diet powdered peanut-oil cake which contains, as analysis showed, ~0.5 mg % choline. The composition of the diet was the following:

Diet I. Peanut-cake meal 30%, casein 6%, lard 18%, sucrose 40%, gelatin 3%, salt mixture 4% (according to Jones and Foster [8] with addition of 0.21 g of sodium fluoride and 0.047 g of alum per 500 g of salt mixture). Vitamins were added in the following amounts per 1 kg of diet: thiamine 2 mg, riboflavin 4 mg, pyridoxine 2 mg, nicotinic acid 2 mg, folic acid 2 mg, calcium pantothenate 10 mg, p-aminobenzoic acid 30 mg, vitamin A 20 I. U. per day, vitamin D 8 I. U. per day.

To free the peanut-cake meal and casein from choline, we extracted them five times for two hours with boiling alcohol. Casein, even when not extracted, contained negligible amounts of choline (0.05 mg per 1 g of dry casein). The alcohol-treated peanut meal contained about 0.15 mg% of choline. Preliminary biological control showed that in animals which received diet I the effects of choline deficiency appeared in the usual period required for the development of choline starvation: after five or six days the characteristic "hemorrhagic degeneration" of the kidney and fatty infiltration of the liver appeared.

A second group of rats (15 animals) received a preparatory five-day feeding of a fully adequate diet II which contained sorbitol as the carbohydrate.

Diet II. Casein 20%, sorbitol 20%, lard 35%, gelatin 3%, salts and vitamins as in mixture I. On the sixth day this diet was changed to a diet analogous to diet I, in which, however, sorbitol was used instead of sucrose (diet III). Since only 20% sorbitol was included instead of 40% sucrose, the amount of fat was correspondingly increased. This mixture had the following composition:

Diet III. Peanut-cake meal 30%, casein 6%, sorbitol 20%, lard 27%, gelatin 3%, salts and vitamins as in diet I.

The third group consisted of 11 animals. Morgan and Yudkin [1] showed that inclusion of sorbitol in the diet caused diarrhea in rats. We also found diarrhea after including sorbitol in the diets shown above. Such diarrhea, since it exhausted our animals, could introduce serious complications into the experiments on the study of choline deficiency, because the characteristic effects of fatty infiltration and kidney change could not develop when the animals were exhausted and their growth disturbed. Therefore, we tried to obtain a preliminary adjustment to sorbitol by steadily raising its content in the food. This was done as follows. The animals received diet II for eight days, with this modification: on the first two days they received 15% sucrose and 5% sorbitol, on the following days the sorbitol content was raised and the sucrose content decreased, so that on the seventh and eighth days the carbohydrate portion of the diet consisted only of sorbitol. At first the animals showed diarrhea, but at the end of this preparatory period it disappeared. At the end of this period (from the ninth day) the animals began to receive diet III. Diarrhea was no longer found in these animals; on the tenth day of the experiment all the rats were killed.

RESULTS

Kidney. One of the most specific effects of choline deficiency is the so-called hemorrhagic degeneration of the kidney [9] which appears in young animals, often even on the third day of choline deficiency and reaches its highest development on the fifth to the sixth day. Macroscopic and microscopic study of the animal kidneys showed that inclusion of sorbitol in the rat diet (the weight of the rats, as mentioned, varied from 40 to 50 g) caused marked inhibition of the development of kidney changes. In the first control group of rats all 16 animals showed changes in the kidneys, in 14 of them severe and in two, a moderate degree of change. In the second group, which received sorbitol from the first day of the experiment, severe kidney change was found in only one, and moderate change in two rats out of fifteen; in the others no change in the kidneys was observed. In the third group, which received the diet with constantly increasing amounts of sorbitol, there was severe kidney change in one rat, moderate in two, and no change in eight rats.

Liver and length of life. The results concerning the liver were different. The characteristic effect of choline deficiency, as already mentioned, is fatty infiltration; in young rats it can sometimes be seen even after 48 hours. The experiment showed that in animals of the second group, the fat content of the liver was much lower than in the first control group, which received sucrose (Fig. 1). The length of life in the animals of both groups was about the same (Fig. 2). In each group only three rats lived to the 11th day, when they were killed. There was no parallelism between length of life and fat content in the liver.

As was shown, diarrhea developed in the animals which received sorbitol and this caused marked exhaustion. When they were killed, their body weight was below that of the controls who died because of lack of choline in the

diet. In choline and protein deficiencies, fat cannot accumulate in the liver* if there is a deficiency of calories [10, 11, 12]. The question of how inclusion of sorbitol in the diet is actually reflected in the fat content of the liver can therefore only be solved in the absence of diarrhea and exhaustion, which occurs in the animals of the third group who had a constantly increasing amount of sorbitol in the diet (in these diarrhea did not occur, the body weight did not fall, and there was no sign of exhaustion (Fig. 3)). On the tenth day, in distinction to the animals of the other group, they remained alive, were active, and appeared healthy; they were all killed on the tenth day. It was shown that in this group of rats, sorbitol did not prevent accumulation of fat in the liver (Fig. 1, third group).

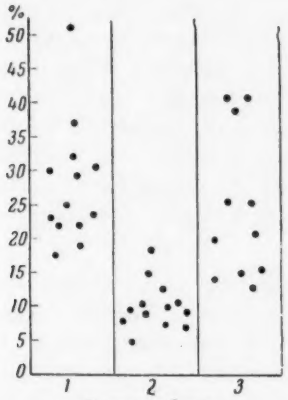

Fig. 1

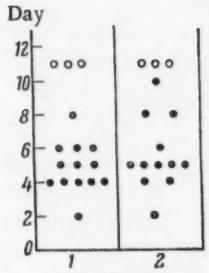

Fig. 2

Fig. 1. Fat content in rat livers:

1) animals receiving choline-free diet with sucrose; 2) animals receiving choline-free diet with sorbitol (without preparatory period); 3) animals receiving choline-free diet with sorbitol (with preparatory period).

Fig. 2. Length of life of experimental rats:

●– rats which died; ○ – rats which lived to 11 days and were killed. 1) sucrose in carbohydrate diet; 2) sorbitol included in diet from first day of experiment.

Since a low content of fat in the liver is found in rats of the second group, in which diarrhea occurred, and exhaustion was observed, and this preventive effect was absent in the animals of the third group, in which there was no exhaustion, it is certain that the low fat content in the animals of the second group is connected with the starvation in calories which comes from the diarrhea. Hence sorbitol, which prevents the pathological process in the kidney, under the conditions of our experiment does not hinder the accumulation of fat in the liver, which is one of the results of choline deficiency (simple lack of calories).

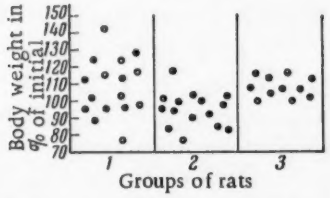

Fig. 3. Weight of experimental animals.
Designations the same as in Fig. 1

Microscopic study of liver preparations establishes the presence of fatty infiltrations in all groups of animals, with a fat content above 16–17%; the intensity of infiltration increases with increased content of fat in the liver.

Treatment of early choline deficiency. We used 50 rats in the experiment. For five days, half of them received choline-free diet I, and beginning on the eighth day this diet was replaced by the sorbitol diet II; sorbitol was included in gradually increasing amounts. The other half of the animals served as controls and continued to receive diet I. The sorbitol diet was given to

* Even in 1938, Best and Ridout [10] showed that when rats obtained less than 34 calories a day, fat did not accumulate in the liver in choline deficiency; fat could accumulate if the rats received 42 calories or more per day.

the animals for varying numbers of days, with a maximum of 25. In the kidneys of most of these animals no change was seen, but the amount of fat in the livers hardly differed from that of the controls. The absence of change in the kidneys, however, could not be attributed entirely to a therapeutic effect of sorbitol, since after ten days of choline starvation, most animals usually showed a spontaneous recovery of the kidney.

DISCUSSION OF RESULTS

These experiments have shown that replacing sucrose by sorbitol hinders the development of a choline deficiency. This is demonstrated by a preventive action with respect to the kidney, but sorbitol under the conditions of our experiment does not prevent changes in the liver. In the experiments of Morgan and Yudkin [1] sorbitol also contributed to an increase in body weight. The different response of the kidney and liver to sorbitol in choline deficiency depends on the different sensitivity of these organs to the lack of choline. The kidney is less sensitive than the liver to this type of deficiency and therefore, to produce a change in it, requires a much greater degree of choline deficit than is required for development of fatty infiltration of the liver. Therefore, changes in the kidney are usually found only in young animals, which are very sensitive to lack of choline, and as they grow, as their need for choline decreases, this effect vanishes spontaneously [9]. We therefore assume that sorbitol assures formation in the organism of only a limited amount of choline, sufficient to prevent kidney changes, but insufficient to prevent accumulation of fat in the liver.

The same results of choline deficiency are found also in cases where sucrose or glucose in a choline-free diet are replaced by starch. This exchange results in the fact that hemorrhagic degeneration of the kidney develops much more rarely in the animals and is less apparent than in the cases where the animal receives sugar; such an exchange of carbohydrates has almost no effect on the intensity of fatty infiltration of the liver [6, 13]. In spite of the limited effect of sorbitol, the possibility of prevention of changes in the kidney in the earliest phases of development of choline deficiency is worthy of consideration because a short-term choline lack (five to seven days) in young animals may be the reason for a typical hypertropic syndrome in a later period in the life of the animal [14, 15].

Experiment also showed that when lactose or galactose served as the sole carbohydrate of a choline-free diet, the animals also did not develop the specific kidney changes [16], and in this case the accumulation of fat in the liver was also prevented [12, 17]. However, in these experiments, the complication of accompanying calorie starvation was evidently not excluded, since galactose and lactose, like sorbitol, cause diarrhea and exhaustion in rats [4, 18].

As to the mechanism of the preventive action of sorbitol, it is most likely that it is related to a change in intestinal flora, which gain or increase in ability to synthesize active methyl groups. Although the main part of the endogenous synthesis of active methyl groups occurs in biosyntheses in the body tissues themselves [19], yet, no doubt, the intestinal flora also share in the formation of "active" CH_3 groups. This is indicated by the fact that some antibiotics can prevent changes in kidney and liver in choline deficiency [20–22].

It should be remembered, however, that as far as starch is concerned, Baxter did not succeed in showing that the preventive effect in this case was actually related to establishing conditions for the intestinal flora which would permit the synthesis of methyl groups [6].

In connection with the question of the possibility of the effect of sorbitol on the condition of the intestinal flora, we must mention a study [23] which shows the effect of the concentration of sorbitol in the diet on the ability of the acetic acid bacteria to oxidize sorbitol to sorbose and the ability of vitamins of the B group to increase the rate of this oxidation.

The data concerning other vitamins also belong to the same category. Experiments have shown that when lactose replaces sucrose completely in the diet, the amount of riboflavin excreted in the urine and feces is doubled [24] and that with such a lactose-containing diet, rats do not need riboflavin and pyridoxine in their food [25]. It is most probable that in all these cases a change in intestinal flora is concerned, the new forms of which have the ability to synthesize certain vitamins. From this point of view, the experiments of Hayashi [26] are of interest; they show that the inclusion of cultures of L. bifidus in rat diet which lacks thiamine but contains thiazole and pyrimidine can greatly increase the excretion of thiamine in the urine.

The data obtained with sorbitol and also the analogous facts concerning other carbohydrates call attention to the role of the intestinal flora. It is well known that many forms of the intestinal flora can synthesize certain vi-

tamins. The facts mentioned above show how certain carbohydrates may furnish conditions for the selective development of forms of the flora which are capable of such syntheses. The peculiarity of sorbitol is that it can assure the synthesis of a much greater number of vitamins which the organism would have to obtain than has been known before for any other carbohydrate.

All these facts again lead us to ideas which agree with the opinion of Mechnikov, but which have gained a new significance. The problem relates not only to the ability of the intestinal flora to form a series of substances which are needed to assure normal metabolism, but also to the possibility that they will intensify the production of some vitamins to such an extent that they will prevent the development of serious pathological processes. So far, however, all these results are only experimental, and it is not yet known how far these desirable changes of the intestinal flora can be carried in humans. Studies should be continued in this direction.

Our results like those of other authors, show that the kind of carbohydrate in an experimental choline-free diet can sometimes affect the course of the experiment to no less degree than the vitamin deficiency itself. This fact should be studied in special experiments and compared with the results obtained by other authors.

SUMMARY

The development of a choline deficiency is hindered if sorbitol is used as the carbohydrate in the choline-free diet.

The replacement of sucrose by sorbitol prevents the development of hemorrhagic degeneration of the kidney, but does not prevent fatty infiltration of the liver under our experimental conditions.

There is reason to assume that sorbitol furnishes conditions for the development of an intestinal flora capable of intensive choline synthesis or synthesis of its precursors.

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A STUDY OF THE PROPERTIES OF 3-PHOSPHOGLYCERALDEHYDE DEHYDROGENASE AND SOLUBLE α-GLYCEROPHOSPHATE DEHYDROGENASE

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In previous work [1] we showed the depressing effect of orthophosphate on the activity of 3-phosphoglyceraldehyde dehydrogenase and the activating action on this enzyme of a series of amino acids and dipeptides. Material which has appeared in the literature up to now allows us to believe that not only substances of a nonphysiological nature, but also compounds which are normally present in the cell can have a depressing action on a number of enzymes. In particular, various inorganic anions have such an action [2-4]. A detailed investigation of the nature of this effect gives new information as to the kinetics of the reaction and the properties of the various enzymes.

In order to explain the problem of the favorable effect of amino acids on the activity of 3-phosphoglyceraldehyde dehydrogenase, we had to make a detailed study of the nature of the suppressing effect of phosphate. One of the most important characteristics of the action of inhibitors in the type of suppression. If the suppression is competitive, it can be thought that the inhibitor blocks active centers in the protein which combine with the substrate; in the contrary case it is concluded that the structure has no relation to the enzyme-substrate complex. Hence a demonstration of the type of suppression permits us to judge the nature of the active centers blocked by inhibitors and also the possible mechanism by which the activating compounds act.

Besides 3-phosphoglyceraldyde dehydrogenase, we selected as an object of study soluble α-glycerophosphate dehydrogenase, or the enzyme of Baranowski [5], which catalyzes the following reaction:

$$\text{PDA}^{*} + \text{DPN H} \rightleftharpoons \alpha\text{-glycerophosphate} + \text{DPN}$$

In its function this enzyme is closely related to 3-PGA dehydrogenase since, along with lactic dehydrogenase, it assures oxidation of the DPN H formed during the oxidation–reduction processes of glycolysis. The wide distribution of α-glycerophosphate dehydrogenase in animal tissues indicates its great value. When the activity of lactic dehydrogenase in the tissues is low or when there is insufficient pyruvic acid, the role of the enzymes which catalyze the dismutation of phosphotrioses becomes fully clear. Although in the tissues of vertebrates the transfer of hydrogen by pyruvic acid predominates and the reaction catalyzed by α-glycerophosphate dehydrogenase is only a "reserve" path, yet in the flying muscles of insects, which work very intensively, the reverse situation is found; with almost complete absence of lactic dehydrogenase, the flying muscles of insects contain a large amount of highly active α-glycerophosphate [6, 7].

Though an enzyme which is closely related functionally to 3-PG dehydrogenase and which carries out an analogous reaction, α-glycerophosphate dehydrogenase has a number of characteristic peculiarities. Thus, it is not inhibited by iodoacetate and cannot be activated by cysteine [5]. The essential difference between the two enzymes is that 3-PGA dehydrogenase is a typical SH enzyme [8], while α-glycerophosphate dehydrogenase does not contain a metabolically active SH group [9]. This difference evidently implies a different type of binding of the substrate when the enzymatic reaction occurs. The purpose of the present work is to study the action of orthophosphate on both enzymes, and, if its action on the Baranowski enzyme is also inhibitory, to compare the type of suppression in both cases.

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* Abbreviations: PDA – phosphodihydrozyacetone, PGA – phosphoglyceraldehyde, DPN – diphosphopyridine nucleotide, DPN H – reduced form of DPN, EDTA – ethylenediamine tetraacetate (versene).

EXPERIMENTAL

Methods of Investigation

3-PGA dehydrogenase and soluble α-glycerophosphate dehydrogenase were isolated from rabbit muscles in crystalline form and purified by the method of Beisenherz et al [10]. The crystals of 3-PGA dehydrogenase (fine plates) were kept in the cold as a suspension in a 2.4 M solution of $(NH_4)_2SO_4$. After two recrystallizations the activity of the preparation was 11,760 M DPN/min per mole of enzyme (pyrophosphate buffer 0.035 M which contained orthophosphate in a concentration of 0.05 M and EDTA in a concentration of 3.10^{-4}M, pH 8.3, temp. 19°). The crystals of α-glycerophosphate dehydrogenase at the moment of formation were rectangular plates, and after standing they took the form of grindstones. The activity of the preparation after one recrystallization was 9340 DPN H/min/mole of enzyme (phosphate buffer, 0.035 M, pH 7.8, temp. 22°). The crystals were kept as a suspension in 1.85 M $(NH_4)_2SO_4$ solution in the cold.

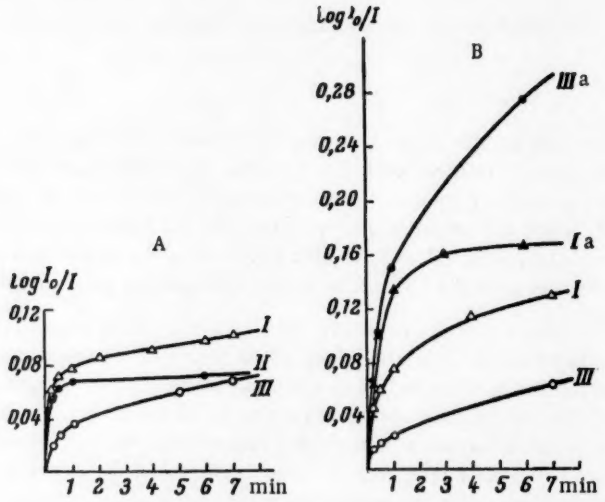

Fig. 1. Effect of different amounts of orthophosphate on the oxidation of 3-phosphoglyceraldehyde.

A sample volume of 3 ml contained: $NaHCO_3$ $5 \cdot 10^{-2}$M, KH_2PO_4 A: I) $5 \cdot 10^{-3}$ M; II) $1 \cdot 10^{-3}$ M; III) $5 \cdot 10^{-2}$ M; B: I) Ia $5 \cdot 10^{-3}$ M; III) IIIa $5 \cdot 10^{-2}$ M. Phosphotriose 5.3 μ mole; DPN 0.14 mg, 3-PGA dehydrogenase 0.004 mg; temp. of incubation 21°, pH 8.6. In samples Ia and IIIa (B) we added histidine to a final concentration of $5 \cdot 10^{-4}$ M, $\log I_0/I$ – increase in optical density at 340 mμ, width of cuvette 1 cm.

For use in the experiments, both preparations were separated from the $(NH_4)_2SO_4$ mother liquor by centrifuging at 0° and 10,000 g and were dissolved in cooled, twice-distilled water. The protein concentration was determined spectrophotometrically [11] and by biuret [10]. Before beginning incubation, the main solution of the enzyme, which contained about 1 mg of protein per 1 ml was diluted with twice-distilled water. As the source for the substrate, phosphoglyceraldehyde (in the case of 3-PGA dehydrogenase) or phosphodihydroxyacetone (in the case of α-glycerophosphate dehydrogenase), we used the mixture of trioses obtained from fructose diphosphate by the method of Meyerhof [12]. The concentration of phosphotriose was determined from the phosphorus which was split after alkaline hydrolysis [13]. In the experiments we used a commercial DPN preparation of about 80% purity; DPN H was prepared by reducing DPN with hydrosulfate [10]. The concentration of DPN H was determined spectrophotometrically. All the solutions were prepared with twice-distilled water; the pH of the solution before the experiment and also the pH of the reaction mixture at the end of incubation were controlled potentiometrically with a glass electrode.

Experiments with 3-Phosphoglyceraldehyde dehydrogenase

The course of oxidation of 3-PGA was followed spectrophotometrically by the increase in amount of reduced DPN. The reaction was started by adding the enzyme.

To obtain the highest activity of 3-PGA dehydrogenase we usually used organic (triethanolamine-HCl [10]; trihydroxymethylaminomethane [14]) or inorganic (pyrophosphate; orthophosphate) buffers which contained versene or cysteine [8, 11]. These compounds had an activating effect on 3-PGA dehydrogenase. We found the reasons for suppression of 3-PGA dehydrogenase and the conditions under which the lost activity could be restored and therefore used a bicarbonate-phosphate buffer without adding special activators.

As Fig. 1 A shows, the course of the reaction depends on the concentration of inorganic phosphate. The optimum value is $5 \cdot 10^{-3}$ M (Fig. 1A, curve I). A fivefold decrease in this amount causes equilibrium at a lower level because of insufficient phosphate (Curve II). An entirely different reason explains the slowing of the reaction in the presence of an excess of phosphate ($5 \cdot 10^{-2}$ M). In this case (Curve III) we find partial inactivation of the enzyme, which is particularly notable in the first minutes of incubation.

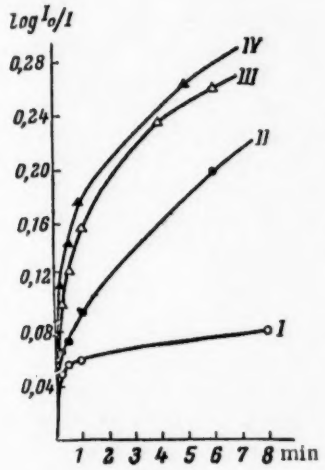

Fig. 2

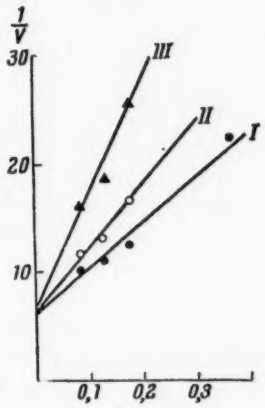

Fig. 3

Fig. 2. Effect of cysteine, histidine, and carnosine on the activity of 3-phosphoglyceraldehyde dehydrogenase.
The sample with a volume of 3 ml contained: $NaHCO_3$ $5 \cdot 10^{-2}$ M; KH_2PO_4 $5 \cdot 10^{-2}$ M; phosphotriose 6.0 μM; DPN 0.14 mg; 3-PGA dehydrogenase 0.007 mg. I) without addition; II) addition of carnosine; III) addition of histidine; IV) addition of cysteine. All additions at a final concentration of $1 \cdot 10^{-4}$ M. Incubation temp. 22°, pH 8.7; log I_0/I – increase in optical density at 340 mμ, width of cuvette 1 cm.

Fig. 3. Relation of activity of 3-PGA dehydrogenase to concentration of orthophosphate with different amounts of substrate.
The sample with volume of 3 ml contained: $NaHCO_3$ $5 \cdot 10^{-2}$ M; KH_2PO_4: I) $5 \cdot 10^{-3}$ M; II) $25 \cdot 10^{-3}$ M; III) $5 \cdot 10^{-2}$ M. DPN 0.2 mg; 3-PGA dehydrogenase 0.008 mg. Length of incubation 15 sec., incubation temp. 23°, pH 8.5.

Thus, under these experimental conditions orthophosphate in a final concentration of $5 \cdot 10^{-2}$ M has an inhibiting effect on 3-PGA dehydrogenase. However, if an activator (aminoacid) is added to the incubating mixture, the relation changes. Now the optimum phosphate concentration is $5 \cdot 10^{-2}$ M (Fig. 1B, curve IIIa), and in a ten-times-smaller amount (I a) is insufficient to give the most active reaction. This implies that phosphate in

high concentration binds active centers of the dehydrogenase and hinders the occurrence of the reaction. The more the phosphate, the less active the enzyme.

The addition of histidine causes freeing of some reactive group so that the activity of the enzyme increases considerably and this indicates the needfor supplying the reaction with the required amount of inorganic phosphate. The activating action of amino acids is certainly not entirely related to lowering the inhibiting action of phosphates. Its role may consist in protecting the dehydrogenase from the action of other inhibitors. However, part of its effect, as Fig. 1 shows, is evidently related to a reaction of phosphate, histidine, and the active centers of the dehydrogenase.

The fact that we are dealing with an effect of phosphate ions and not with impurities which they contain, was shown in the following way. The KH_2PO_4 preparation was treated with 8-hydroxyquinoline [15], to free it from traces of heavy metals andwas then recrystallized. The effect of the preparation purified in this way did not differ from that of the untreated KH_2PO_4.

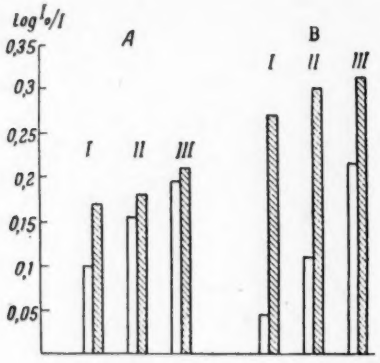

Fig. 4. Effect of histidine on the activity of 3-phosphoglyceraldehyde dehydrogenase at different phosphotriose concentrations.
A sample with volume 3 ml contained: $NaHCO_3$ $\cdot 5\cdot10^{-2}$ M; KH_2PO_4 $5\cdot10^{-3}$ (A) or $5\cdot10^{-2}$ M (B). Phosphotriose: I) 5.7 μM, II) 11.5 μM, III) 23.0 μM. DPN 0.14 mg. 3-PGA dehydrogenase 0.004 mg. Incubation period 6 min, temp. 20°, pH 8.3. White columns – without addition, cross-hatched columns – added $5\cdot10^{-4}$ M of histidine; log I_0/I increasing optical density at 340 mμ. Width of cuvette 1 cm.

Our experiments showed that different amino acids have different ability to show a protective action in maintaining the high level of activity of PGA dehydrogenase. For example, glycine in a concentration of $1\cdot10^{-4}$ M restores only 5% of the activity of the dehydrogenase, while the same concentration of histidine raises the level of the reaction more than three times (Fig. 2). The effect of this amino acid is of interest, since it is almost equal to the action of an equimolecular amount of cysteine. It is possible that along with the SH groups, the imidazole residue also has a decisive role in the activity of 3-PGA dehydrogenase. As Fig. 2 shows, carnosine has an effect as evident as that of histidine.

Since we noted the inhibiting effect on 3-PGA dehydrogenase of a large concentration of phosphate, we tried to determine to what type this inhibition belonged. If there existed a competitive relation between the substrate and the phosphate, then the activating effect of amino acids should be explained by their presence supplying an easier, unhampered reaction of PGA and the protein.

In order to solve this question we ran parallel incubations in three buffers with different phosphate concentions and measured the rate of reaction when we added increasing quantities of phosphotriose. The results of the experiment are illustrated in Fig. 3. On the ordinate is plotted the inverse of the reaction rate and on the abscissa the inverse of the substrate concentration. The distribution of the curves which correspond to different concentrations of phosphate buffer shows that there is a competitive relation between the substrate and the phosphate. In other words, phosphoglyceraldehyde can protect the dehydrogenase from inhibition by phosphate.

The lower the concentration of substrate, the stronger is the inhibition. PGA evidently competes with phosphate for the definite active centers on the protein. The activating role of amino acid can include the protection of these centers from phosphate and the assurance of more rapid reaction of the enzyme with the substrate. In this case, the effect of the amino acid should decrease with increasing concentration of phosphoglyceraldehyde, as Fig. 4 shows. In the buffer with a phosphate concentration of $5\cdot10^{-3}$ M without addition of histidine, the reaction rate is higher than in the buffer which contains $5\cdot10^{-2}$ M KH_2PO_4. However, this difference levels off with increasing amounts of phosphotriose and at an excess (23 μM in the sample), it disappears altogether. The addition of histidine to both buffers has a greater effect on the reacion when the phosphotriose concentration is lower.

An increased amount of DPN added to the incubation mixture causes a considerable rise in reaction rate, as is clear from a comparison of the results of the experiments given in Fig. 4 and the table. However, this does not occur under all conditions. When the phosphotriose concentration is small (5 μM in the sample), the competition with the phosphate is sharpest for the active centers of the dehydrogenase, and the addition of increased amounts of DPN does not assure a better course for the reaction. When the suppressing action of the phosphate is less clearly shown (in the presence of histidine or of a large amount of phosphotriose) the effect of suplementary amounts of DPN becomes very obvious (Table).

TABLE

Effect of Histidine on Activity of 3-PGA Dehydrogenase Under Different Conditions

A sample with volume 3 ml contained: $NaHCO_3$ $5 \cdot 10^{-2}$ M; DPN 0.5 mg; 3-PGA dehydrogenase 0.003 mg; histidine $1 \cdot 10^{-3}$ M; KH_2PO_4 and phosphotriose in concentrations given in the table. Incubation period 6 min; temp. 20°; pH 8.5. Results expressed in log I_0/I (increase in optical density at 340 mμ). Width of cuvette 1 cm.

μM of phosphotrioses in sample	$5 \cdot 10^{-3}$ M KH_2PO_4		$5 \cdot 10^{-2}$ M KH_2PO_4	
	without addition	with histidine	without addition	with histidine
5,0	0,08	0,38	0,04	0,62
20,0	0,32	0,51	0,16	0,70
40,0	0,43	0,55	0,42	0,80

Thus, suppression of dehydrogenase activity by phosphate occurs only when compounds which compete with it for active centers of the enzyme protein are absent or present in small amount. In the presence of large concentrations of orthophosphate, as Velick and Hayes [14] showed, there is nonspecific binding of phosphate ions by 3-PGA dehydrogenase. It is so considerable that it leads to a shift in the isoelectric point of the protein toward the acid side. At this, evidently, there can occur reversible blocking of specific groups which are responsible for binding the substrate. At the present time, most authors agree that 3-PGA reacts with dehydrogenase by its aldehyde group, and then its oxidation follows with formation of an acyl enzyme. However, the question of the primary binding of the substrate to the dehydrogenase cannot be considered solved.

The different relationship of phosphoglyceraldehyde and glyceraldehyde to dehydrogenase lies in the strength of the bond of the phosphate residue with the dehydrogenase. Some authors [8, 16] assume that the primary bond between dehydrogenase and substrate is through the phosphate group. When they find a suppression of the activity of dehydrogenase by high concentrations of arsenate, these authors tend to treat it as due to competition between the arsenate and the phosphate groups of phosphoglyceraldehyde. An analogous effect is evidently observed in the presence of a high concentration of phosphate. Thus the results of our experiments confirm the possibility that the primary reaction of phosphoglyceraldehyde with the enzyme is through the phosphate group.

The substrate for the action of α-glycerophosphate dehydrogenase is also a phosphotriose. We thought it possible to consider that in this case the phosphate residue would play a part in the reaction of phosphodihydroxyacetone with the enzyme. In this connection it was interesting to study the effect of relatively high concentrations of orthophosphate on the Baranowski enzyme.

Experiments with α-Glycerophosphate Dehydrogenase

The activity of α-glycerophosphate dehydrogenase was studied spectrophotometrically, measuring the increase in DPN H during incubation. Our experiments on testing the activity of α-glycerophosphate dehydrogenase in different buffers showed that in 0.03 M phosphate buffer, the reaction rate was somewhat lower than in a veronal buffer of the same concentration. The unfavorable effect of phosphate ions became clearer if we used a greater concentration of buffer. As Fig. 5 shows, the reaction rate in samples which contain orthophosphate at a final concentration of 0.21 M is appreciably lower than in samples where the phosphate concentration is 7 times less.

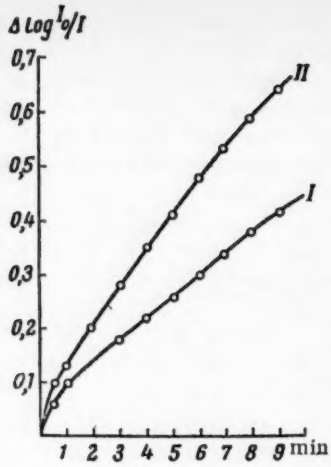

Fig. 5. Suppressing action of orthophosphate on the activity of α-glycerophosphate dehydrogenase.
Composition of sample: 01 ml DPN H(0.4 μM), 0.5 ml phosphotriose solution (1.0 μM PDA), 2.3 ml phosphate buffer, pH 7.7; (final concentration of phosphate in sample: I – 0.21 M, II – 0.03 M), 0.1 ml α-glycerophosphate dehydrogenase (0.002 mg protein), temp. 18°, Δ log I_0/I – decrease in optical density at 340 mμ.

Thus, the inhibiting action of a relatively high orthophosphate concentration is characteristic not only for 3-PGA dehydrogenase, but also for the Baranowski enzyme. Is it the same type of inhibition in both cases? To explain this question we had to consider the following. α-Glycerophosphate dehydrogenase, as Fig. 6 shows, has a rather narrow zone of optimum pH. A comparatively small variation in size of pH on both sides of this zone leads to a marked fall in enzyme activity. In the presence of a high concentration of orthophosphate, part of this is evidently bound to the protein. The result of this can be a change in the isoelectric point and a shift in the optimum pH of the enzyme. The inhibiting action of the phosphate in this case would be ascribed to the fact that the sample with a high buffer concentration would be outside the optimum zone and thus the enzyme activity would be decreased.

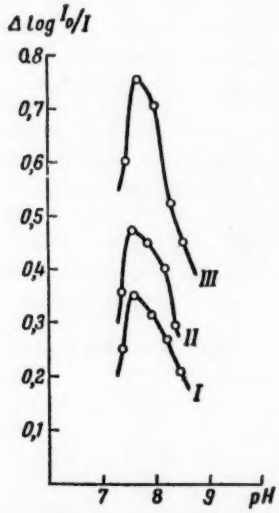

Fig. 6. Optimum pH of α-glycerophosphate dehydrogenase in phosphate buffers of different concentrations.
Composition of sample: 0.1 ml DPN H (0.4 μM), 0.5 ml of phosphotriose solution (1.0 μM PDA), 2.3 ml of phosphate buffer, 0.1 ml of α-glycerophosphate dehydrogenase (0.002 mg protein), temp. 21°, incubation period 8 min. Final concentration of orthophosphate in the sample I – 0.5 M, II – 0.2 M, III – 0.033 M. Δ log I_0/I – decrease in optical density at 340 mμ.

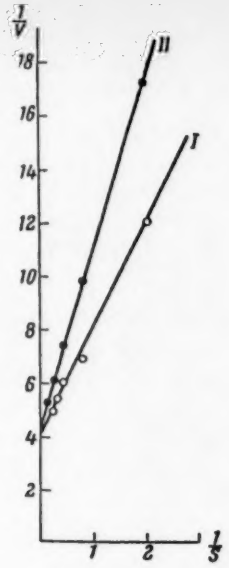

Fig. 7. Relation of activity of α-glycerophosphate dehydrogenase to concentration of phosphate buffer at different amounts of substrate.
Composition of sample: 0.1 ml DPN H (0.4 μM), 0.5 ml phosphotriose solution (0.5, 1.2, 2.5, 3.7, 0.5 μM PDA in sample), 2.3 ml of phosphate buffer, pH 7.7, 0.1 ml α-glycerophosphate dehydrogenase (0.002 mg protein). Final concentration of orthophosphate in sample: I – 0.03 M; II – 0.21 M, temp. 18°, incubation period 60 sec.

In order to test the correctness of this assumption, we carried out an incubation in phosphate buffer solutions of different concentrations. Comparison of the rate of reaction at different pH values (Fig. 6) allowed us to draw two conclusions: 1) orthophosphate shows an inhibiting action on α-glycerophosphate dehydrogenase; the inhibition is stronger the higher the concentration of buffer; 2) increasing the phosphate concentration 15 times does not cause a marked shift in the optimum pH of the enzyme. Thus the action of phosphate must be viewed as blocking the centers on the enzyme protein which are important for its catalytic activity. Do these centers take part in binding the substrate? To settle this we followed the relation of the degree of inhibition of dehydrogenase activity to concentration of phosphodihydroxyacetone.

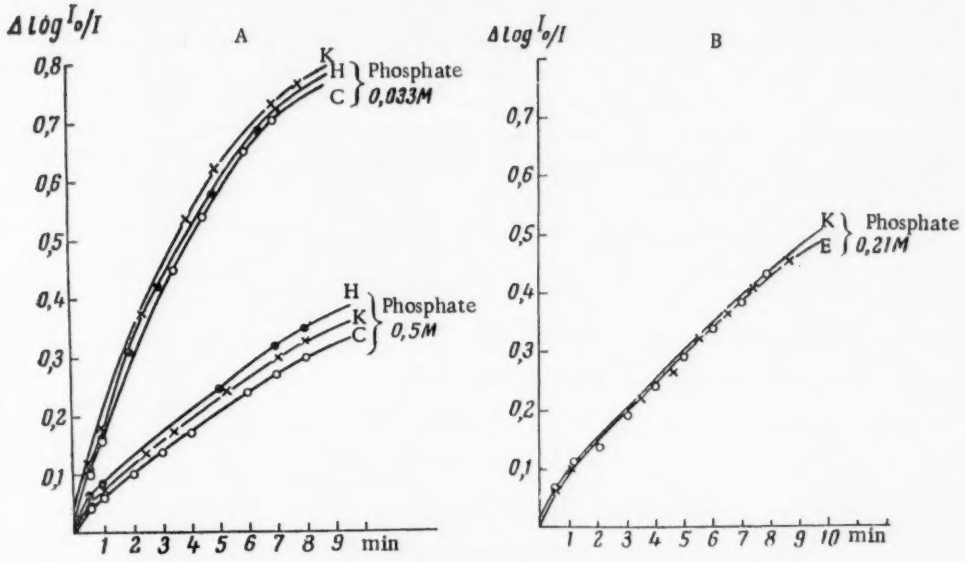

Fig. 8. Activity of α-glycerophosphate dehydrogenase in the presence of histidine, cysteine, and EDTA.
Composition of sample: 0.1 ml DPN H (0.4 μM), 0.5 ml phosphotriose solution (1.0 μM PDA), 2.3 ml phosphate buffer, 0.1 ml α-glycerophosphate dehydrogenase (0.002 mg protein). Temp. 20°, A: 0.1 ml H_2O, (K), histidine (H), cysteine (C) at a final concentration of $1.5 \cdot 10^{-3}$ M, pH 7.7. B: 0.1 ml H_2O (K), EDTA (E) at a final concentration of $3 \cdot 10^{-3}$ M, pH 7.8, $\Delta \log I_0/I$ – decreasing optical density at 340 mμ.

The results of the experiment are given in Fig. 7. The inverse rate of reaction is plotted against the inverse concentration of substrate. The course of the curves indicates that the inhibition has a competitive character. Hence the effect of orthophosphate on the Baranowski enzyme is analogous to its effect on 3-phosphoglyceraldehyde dehydrogenase (Fig. 3). In both cases the phosphate evidently competes with the substrate for active centers on the protein.

In this connection it seemed probable that the primary binding of phosphodihydroxyacetone to the α-glycerophosphate dehydrogenase, just as the binding of 3-PGA to its dehydrogenase, occurred through the phosphate group. In the latter case, the active centers in all probability are SH groups; this explains the effect of cysteine on the activity of 3-PGA dehydrogenase, especially the full removal of the inhibiting effect of phosphate ions in the presence of this compound in our experiments (Fig. 2).

The fact is different with α-glycerophosphate dehydrogenase. It is characterized by the absence of SH groups, which are important for enzyme activity. Therefore, the results of the experiments shown in Fig. 8A are quite understandable. The addition of cysteine (and histidine) has no effect on the enzyme activity, but it removes the inhibiting action of large concentrations of phosphate. Fig. 8B shows that the addition of versene also does not affect the rate of the reaction. Hence, the assumption is excluded that the action of phosphate can be connected with the presence of contaminating heavy metals which inhibit the enzyme. As in the case of 3-PGA dehydrogenase, there is here, evidently, a non-specific binding of the phosphate ions by the protein.

Different groups can be blocked, but as our experiments showed, the inhibition of enzyme activity is the result of binding the centers which combine with the substrate. While in the case of 3-PGA dehydrogenase, in addition to the substrate, a number of amino acids also have a protective action, yet for α-glycerophosphate dehydrogenase no such activators have yet been found. At the present time we also lack information as to the nature of the active centers of this enzyme.

The inhibition of the activity of these two enzymes by orthophosphate is evidently an example of a widely distributed inhibiting effect of salts on enzymes of very different types. It is possible that the relations of the concentration of inhibiting agent, substrate, and activator play a part in regulating the rate of enzymatic processes.

SUMMARY

Orthophosphate in concentrations of 10^{-2} to 10^{-1} M has an inhibiting effect on the activity of 3-phosphoglyceraldehyde dehydrogenase and soluble α-glycerophosphate dehydrogenase. In both cases, the inhibition has a competitive character. Some amino acids (cysteine, histidine), added in small concentrations ($1 \cdot 10^{-4}$ M) fully remove the inhibiting action of phosphate for 3-phosphoglyceraldehyde dehydrogenase. In the case of α-glycerophosphate dehydrogenase the addition of amino acids does not restore the activity of the enzyme when it has been reduced in a phosphate buffer.

We express deep thanks to Corresponding Member AN SSSR S. E. Severin for his guidance in this work.

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THE HEXOKINASE ACTIVITY OF SKIN

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Our previous studies [1, 2] showed the presence of an active amylase and a slightly active phosphorylase in skin. As a result of the action of the skin amylase, maltose and glucose were formed from polysaccharides [3].

According to present ideas, the utilization of glucose begins with its phosphorylation, which requires hexokinase. In the course of the hexokinase reaction, glucose is phosphorylated with ATP phosphorus to form glucose-6-phosphate.

Hexokinase was discovered by Meyerhof [4] in yeast; then this enzyme was also detected in animal tissues: in liver [5, 6, 7], in the brain of mature and young animals [8, 9], in muscle [10, 11, 12].

The path of transformation of glucose in the skin has not been studied up to now. In our work we have looked for the presence of hexokinase in skin.

METHODS

There are two methods for determining hexokinase: by the amount of residual glucose after the hexokinase reaction, or by the amount of phosphorus transferred from ATP to glucose.

On the basis of the work of Bekina and Petrova [5], we chose the latter method as the more exact.

In work with unpurified enzymes, high activity of the phosphatases can hinder full appearance of the hexokinase reaction. In our work we prevented their action with sodium fluoride.

The study was carried out with the skin of laboratory animals. A weighed sample of skin was freed from subcutaneous fat cells and ground in the cold with 2 ml of water per g of skin. After standing for 20 min, the homogenate was centrifuged. 1 ml of the extract obtained in this way was added to 4 ml of an incubation mixture which consisted of a final glucose concentration of 0.003 M; ATP, 0.005 M; NaF, 0.05 M; $MgCl_2$, 0.006 M; pH of the incubation mixture 7.2–7.3. Incubation period 20 min at 37°.

The protein was precipitated with an equal volume of 5% TCA. In the control tests, TCA was added to the original extract.

Inorganic phosphorus, P_i, was determined colorimetrically in the photoelectric colorimeter by reaction with the molybdenum reagent.

The ATP content was determined by the amount of P_i liberated after 10 min hydrolysis in N HCl at 100°, using the same method.

RESULTS

In studying the hexokinase activity of the skin by decrease in ATP it was necessary to be sure that when we incubated our extracts, the content of no compound other than ATP, which contained easily hydrolyzed phosphorus, was changed.

In Table 1 we give data on the determination of P_i and easily hydrolyzed phosphorus P_h with incubation without adding ATP but with addition of all the other components of the experimental mixture.

Table 1 shows that the incubation mixture which we studied did not contain compounds with easily hydrolyzed phosphorus and such compounds were not formed during incubation.

TABLE 1

Easily Hydrolyzed Phosphorus Content in Extract of the Skin of the Guinea Pig. Experimental conditions: extract 1:10; glucose – 0.003 M; NaF – 0.05 M; $MgCl_2$ – 0.006 M, ATP not added; pH 7.3–7.4. Incubation 20 min at 37°

P_i in μg/sample		$P_i + P_h$ in μg/sample		P_h in μg/sample		Δ^{P_i}
before incubation	after incubation	before incubation	after incubation	before incubation	after incubation	
129,0	132,0	129,0	132,0	0	0	+3,0
217,0	217,0	217,0	217,0	0	0	0
205,2	206,0	205,2	206,0	0	0	+0,8
176,5	180,0	176,5	180,0	0	0	+3,5
152,4	152,5	152,5	152,5	0	0	0

TABLE 2

Effect of Dilution of the Extract on Hexokinase Activity of Guinea-Pig Skin. Experimental conditions: glucose – 0.003 M; NaF – 0.05 M; $MgCl_2$ – 0.006 M; ATP 0.005 M; pH 7.3–7.4. Incubation 20 min at 37°. Value of P is given in all samples

Dilution 1 : 20						Dilution 1 : 10					
P_i, μg		P_h, μg		P transferred to glucose		P_i, μg		P_h, μg		P transferred to glucose	
Before incubation	After incubation	Before incubation	After incubation	μg	%	Before incubation	After incubation	Before incubation	After incubation	μg	%
340	325	695	700	—	—	267	270	375	345	30	8,0
195	225	645	795	—	—	220	205	865	815	50	5,7
175	185	570	580	—	—	205	205	560	480	80	14,2
220	225	580	595	—	—	252	250	540	510	30	5,5
175	185	810	830	—	—	175	185	845	735	80	9,8

In a special series of experiments we studied the effect of dilution of the extract on hexokinase activity of the skin.

For the hexokinase reaction, most authors [5, 8, 13] have used extracts with a final dilution of 1:20.

As Table 2 shows, the skin extracts with a dilution of 1:20 either have no hexokinase activity, or only a very slight one and so are not suitable for this study.

A more favorable dilution is 1:10 at which 5.5–14% of the phosphorus is transferred to the glucose, and we used this concentration in all our later experiments.

The hexokinase activity was determined in the skin of rabbits, guinea pigs, and white mice.

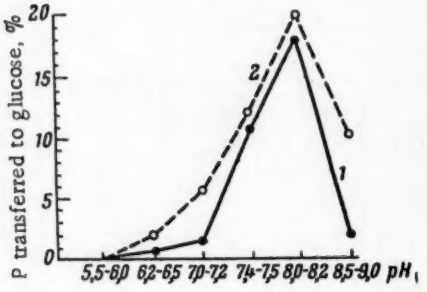
The data of Table 3 show that the skin of these animals has hexokinase activity.

Under the influence of hexokinase the skin of rabbit and guinea pigs transfers from 5 to 18% of the ATP phosphorus to glucose, which is 25-80 μg P in a sample.

The hexokinase of white-mouse skin catalyzes the transfer of 6-34% P.

The optimal pH for this reaction differs for different tissues. One author considers that hexokinase is most active at pH 7.2-7.4 [8]. In muscle, hexokinase shows optimum activity at 8.0-8.1, in rhabdomyoblastoma, at pH 7.7 [12]; in brain [14], at 7.4-7.6.

The physiological value of pH for skin, according to the data of Kaplanskii [15] is 7.2-7.4.



Effect of pH on hexokinase activity of skin. Average data from 8 experiments. 1) guinea pig; 2) rabbit.

TABLE 3

Hexokinase Activity of the Skin of Rabbits, Guinea Pigs, and White Mice. Experimental conditions: extract 1:10;* glucose—0.03 M; ATP—0.005 M; NaF—0.005 M; $MgCl_2$—0.006 M; pH 7.3-7.4. Incubation 20 min at 37°

Guinea pig		Rabbit		White mouse	
P transferred to glucose					
µg/sample	%	µg/sample	%	µg/sample	%
42	15	66	18,3	25	6,6
70	13,3	49	6,6	20	5,7
47	17,6	14	5,0	40	11,1
30	8,0	65	8,6	55	16,6
50	5,7	40	8,0	45	13,9
80	9,8	112	11,05	30	9,0
80	14,2	57,2	5,8	85	21,2
70	11,8	45	8,2	55	13,2
25	5,0	45	6,2	85	34
—	—	35	5,0	—	—

*In the studies on white mice the source of the hexokinase was a skin homogenate.

However, as the following series of our experiments showed, under these conditions skin hexokinase did not show its maximum activity.

We carried out an investigation with a medinal buffer at pH values from 5.5 to 9.0.

The results, given in the figure, show that the greatest hexokinase activity of skin is at pH 8.0–8.2.

Under these conditions 17.38% of the P on the average, is transferred to glucose in guinea-pig skin, and 19.75% P in rabbit skin.

Thus, our investigation shows that in the skin of rabbits, guinea pigs, and white mice, hexokinase is present. Hence, in skin, as in other tissues, the use of glucose can begin with its phosphorylation.

The activity of hexokinase increases with change of pH to 8.0–8.2 and is fully inhibited at pH 5.5–6.0.

SUMMARY

We have shown that aqueous extracts of the skin of rabbits, mice, and guinea pigs have hexokinase activity. The optimum dilution of the extracts for observing hexokinase in the skin is 1:10. The optimum pH of the hexokinase is 8.0–8.2. At pH 5.5–6.0 the hexokinase activity is absent.

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STEROLS OF HEN EGGS. 7-(α)-HYDROXYCHOLESTEROL AND ITS CONTENT IN EGGS WITH RELATION TO THE TIME OF INCUBATION

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One of the many unsolved questions of cholesterol metabolism is the great accumulation of cholesterol in the yolk of hens' eggs, where its content reaches 1.7%. It might be thought that cholesterol is the source from which the developing embryo draws the cholesterol for the formation of its tissues and organs. However, Stokes, Fish, and Hickey [1] showed that the embryo of the hen's egg can synthesize cholesterol from added labelled acetate. This gives a basis for the assumption of the presence of sterol metabolism during the development of the chicken embryo. Actually, these authors reported [3] that they isolated from the 12-14-day-old chicken embryo two sterols as p-iodobenzoates: desmosterol and a desmosterol isomer.

We also undertook a systematic study of sterol metabolism in the chicken egg and in the chicken embryo. In the present communication we give data which show that in the chicken egg, besides cholesterol, there is also 7-(α)-hydroxycholesterol, the content of which changes in the egg during the development of the embryo.

EXPERIMENTAL

Isolation of 7-(α)-hydroxycholesterol

Starting from the assumption that the products of sterol metabolism in the egg will be present in a very small amount, we used for each study thirty White Leghorn eggs, obtained from the Leningrad hatchery. The eggs were studied before incubation and at 3, 9, and 12 days incubation.

Methods. The contents of the eggs were removed from the shells and ground as carefully as possible in a porcelain mortar. Acetone was gradually added during the grinding until it reached 5-6 times the volume of original material. After careful stirring, the mixture was left until the next day, and then the precipitate was filtered off and again ground with the same amount of acetone. Such treatment of the precipitate with acetone was repeated 5-6 times, the filtrates were combined, the acetone was completely distilled off, and the residue was repeatedly extracted with ether. The resulting ether solution was dried with sodium sulfate, and then the ether was distilled off. The residue was an oil, the amount of which, from the treatment of 30 eggs, was usually 170-180 g. The oil was saponified in a nitrogen atmosphere with 10% NaOH in alcohol solution by heating on a boiling-water bath for 1 hour, after which the alcohol was completely distilled off. As the alcohol distilled, water was added to the solution so that the volume of the solution did not change. After the solution had cooled, the precipitated soap was filtered, the filtrate was repeatedly extracted with ether, and the residual soap was dissolved in alcohol; to the hot alcoholic solution we added a hot concentrated solution of barium chloride to full precipitation of the soap as the barium salt. The precipitate was filtered and treated with hot alcohol for full removal of adsorbed sterols. The alcohol was distilled from the resulting alcoholic solution and the residue was extracted many times with ether to remove the unsaponified lipid fraction. (Removal of these substances without precipitation of the fatty acids by barium salts was not possible). The resulting ether solution was combined with the ether solution obtained by treating the filtrate after removal of the soaps from the water solution, and the ether was dried with sodium sulfate. The ether was distilled from the solution down to a volume of 80-100 ml and the residue was diluted with acetone until no more soap precipitated; the soap was filtered off. The solvent was fully distilled from the filtrate, and the residue which was obtained was crystallized from alcohol for separation of

the cholesterol. The isolated cholesterol was crystallized once more from alcohol, weighed, and its melting point determined; this was usually 146-148°. After separation of the cholesterol the alcohol was distilled from the filtrate and the residue dried in a vacuum desiccator and submitted to chromatographic separation on Al_2O_3, as described in previous work [3].

The adsorbent was a preparation from Glavkhimreactiv, the amount of which taken was 10-15 times more than that of the substance to be analyzed. From the benzene-ether fraction (50:50), samples 1-12 and the ether fraction, samples 1-20, we isolated cholesterol, purified it as indicated, and added it to the already-weighed quantity.

TABLE 1

Amount of Cholesterol and Hydroxycholesterol Isolated from Hens' Eggs at Different Stages of Incubation
Each separation was carried out on 30 eggs

Period of Incubation	Cholesterol, g	Hydroxycholesterol, mg
Before incubation	5	14.3
3 days incubation	5.8	2.0
9 days incubation	6.0	57.5
12 days incubation	6.5	38.3

Hydroxycholesterol was isolated from the ether-methanol (99:1) fraction, samples 1-10, and gave characteristic reactions for a hydroxycholesterol compound with antimony trichloride and the Lifschutz reagent. In order to obtain a purer hydroxycholesterol, we submitted all the fractions which gave these positive reactions to the same chromatographic purification. The results of this study are given in Table 1.

The hydroxysterol obtained from eggs at different periods of incubation appears as a white substance, homogeneous in appearance, and giving an intense blue color with antimony trichloride and the Lifschutz reagent. For final identification, all the hydroxysterol fractions were combined and crystallized. The substance separated as a white, amorphous material from all the solvents which we tried. Since we knew from the literature that only 7-(α)-hydroxycholesterol occurs as an amorphous substance which has never been obtained as a crystal, this fact shows that the substance which we isolated is 7-(α)-hydroxycholesterol. To confirm this, we prepared the characteristic derivative, the dibenzoate.

Obtaining the dibenzoate. 40 mg of the substance was dissolved in 1 ml of dry pyridine, 0.3 ml of freshly distilled benzoyl chloride was added dropwise, and the mixture was allowed to stand for a day at room temperature, after which it was cooled and diluted with water. The precipitated oil was extracted with ether, the ether solution was washed with water, with dilute sulfuric acid, again with water, was dried over sodium sulfate, and after distillation of the ether, the residue was dried in a vacuum desiccator and chromatographed on Al_2O_3. From the petroleum ether and petroleum ether-benzene (1:1) fractions we isolated 7-(α)-hydroxycholesterol dibenzoate, which after four crystallizations from methanol melted at 170-171°.

TABLE 2

Content of 7-(α)-Hydroxycholesterol in Eggs (mg) Depending on Length of Incubation

Experiment No.	Length of incubation, days					
	0	3	6	9	12	15
1	0.12	0.24	0.14	0.86	0.40	0.26
2	0.14	0.28	0.15	1.00	0.36	0.28
3	–	–	–	0.84	–	0.29

A sample of this dibenzoate mixed with the dibenzoate of 7-(α)-hydroxycholesterol obtained by the process which one of us described earlier [4] gave no lowering of the melting point, but melted at 170-171°.

Content of 7-(α)-Hydroxycholesterol in Eggs, Depending on the Length of Incubation

The data of Table 1 show that the amount of 7-(α)-hydroxycholesterol changes with the time of incubation of the egg. However, the method of isolation which we used, though very suitable for preparation of the substance, is too complex for its quantitative determination. For the latter purpose we worked out the following colorimetric method for the 7-(α)-hydroxycholesterol in eggs.

The egg was treated with acetone as already described. After distillation of the ether solution, the residue was dissolved in 100 ml of methanol, transferred to a separatory funnel and shaken with 100 ml of petroleum ether for several minutes. After the layers had separated and the petroleum ether fraction had been removed, the meth-

anol solution was once more shaken in the separatory funnel with 50 ml of petroleum ether and this solution again separated from the methanol solution. As a result of such treatment, the main mass of fat and cholesterol was in the petroleum ether and all the hydroxycholesterol with small admixtures of fat and cholesterol was in the methanol solution. The residue after distillation of the methanol was dried in a vacuum desiccator and submitted to chromatographic separation on Al_2O_3 as already described. The hydroxycholesterol was separated in the ether-methanol (99:1) fraction.

After distillation of the solvent the residue was dissolved in 10 ml of alcohol. For the quantitative determination of 7-(α)-hydroxycholesterol, we took 0.025-0.2 of the total volume of the solution, depending on the amount of the substance in the eggs. After evaporation of the alcohol, the residue was dried in a vacuum desiccator and 6 ml of the Lifshits reagent was added to it; it was carefully mixed, and after 10 min was determined in the photoelectric colorimeter. The amount of sterol was determined from a calibration curve obtained on the same apparatus with concentrations of 7-(α)-hydroxycholesterol from 0.01 mg to 0.05 mg. The content of 7-(α)-hydroxycholesterol was determined for eggs before incubation and at 3, 6, 9, 12, and 15 days of incubation. The results are given in Table 2.

DISCUSSION OF RESULTS

The method for quantitative determination of 7-(α)-hydroxycholesterol which we have described in this paper is considerably simpler than the method which was worked out earlier by one of us with Panyukov and Shpats [3], where it was shown that in the treatment of tissues for isolation of 7-(α)-hydroxycholesterol, its formation from cholesterol was excluded. This is indicated by the good agreement of the results of determination of 7-(α)-hydroxycholesterol in eggs at the same period of incubation.

The facts which we have presented allow us to consider that 7-(α)-hydroxycholesterol is a natural product of sterol metabolism in chicken eggs and developing embryos, and is not an artificial product arising from cholesterol during the process of treatment of the eggs. The results in Table 2 show that the content of 7-(α)-hydroxycholesterol is sufficiently constant in eggs at the same stage of incubation, but changes as the embryo develops, reaching a maximum on the ninth day of incubation of the egg, after which the content of this substance begins to decrease. We can assume that this compound plays a part in the complex processes of development of the chicken embryo, although its role and connection with the processes of metabolism of cholesterol remain unsettled.

SUMMARY

We have isolated, from hens' eggs before incubation and at 3, 9, and 12 days of incubation, 7-(α)-hydroxycholesterol and have obtained its dibenzoate with m. p. 170-171°. We have given a colorimetric method for quantitative determination of the sterol in eggs. By the use of this method we have established that the content of 7-(α)-hydroxycholesterol is constant in eggs at the same period of incubation, but that it changes during development of the embryo, reaching a maximum on the ninth day of incubation and then gradually decreases in later stages of incubation of the egg.

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THE FIXATION OF PROTEASES BY WOOL

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As is known, wool and silk fibroin have considerable stability toward proteases. They are also insoluble in the usual protein solvents. These properties permit us to study their ability to fix proteases. Aside from a number of practical considerations, which we have followed up, we wish to study a number of questions concerning the laws of formation of enzyme-substrate complexes [1].

In the experiments we used wool from coarse-wooled sheep, which was used for manufacturing felt.* The wool was washed with hot distilled water and soap, and defatted with acetone, alcohol, and ether. After air drying, the wool was cut up with scissors to a finely divided state. Silk fibroin was also cut up. We used lyophilically dried crystalline preparations of trypsin, chymotrypsin, and pepsin, prepared by the method of Northrop and Kunitz [2, 3], and also an amorphous preparation of papain. The concentration of trypsin and chymotrypsin in the solutions was determined by the intensity of hydrolysis of casein [4, 5], and the concentration of pepsin and papain by the hydrolysis of crystalline beef hemoglobin [6].

For the study of the protease activity of trypsin we used a preparation of crystalline albumin from horse serum. In the experiments we used 0.066 M phosphate buffer, pH 7.9; 0.02 M citrate buffer, pH 3.9; and 0.1 M borate buffer, pH 8.0. The latter was used when calcium chloride was employed.

The experiments with trypsin and chymotrypsin were run as follows. To 300 mg of minced wool or silk fibroin in a centrifuge tube, we added 4 ml of cooled 0.05% solution of the enzyme in a phosphate (pH 7.9) or citrate (pH 3.9) buffer. The wool was stirred with the enzyme solution for 7 min with ice cooling. At the end of this period the wool was separated from the enzyme solution by centrifuging at 3000 rpm and the amount of unfixed enzyme in the centrifugate was determined from the hydrolysis of casein after 25-fold dilution with 0.0025 N HCl. The wool was washed once with 0.0025 N HCl, adding 6 ml, with account taken of the remaining enzyme solution (usually about 1 ml). In this case we also determined the concentration of enzyme which dissolved after a twofold dilution before determining the activity. After the washing, the enzyme fixed by the wool was liberated with the aid of various substances. For this purpose, the samples with the wool were brought to 10 ml with the solution to be studied, with later tenfold dilution before determining the enzyme activity.

For determining the effect of cysteine on trypsin and chymotrypsin, we carried out experiments with determination of the activity of the enzyme solutions in the presence of cysteine. We also determined autolysis of the enzyme by the appearance of peptides which were not precipitated by TCA. For each 2 ml of enzyme solution, we added 3 ml of 5% TCA solution. The resulting precipitate was removed by centrifuging and the optical density of the centrifugate was determined at 280 mμ.

The results of the experiments on fixing proteases by wool and silk fibroin are given in Table 1. All the experiments, as a rule, gave well-reproducible results. As the data show, trypsin and chymotrypsin mixed with wool or fibroin (ratio of enzyme to substrate 1:150) are well fixed by them. Pepsin and papain are not fixed by the above substrates.

* We carried out some experiments with wool from fine-wooled sheep, but in this case trypsin and chymotrypsin were poorly fixed.

TABLE 1

Fixing of Proteases by Wool and Silk Fibroin

Enzyme	Amount of enzyme in starting solution, μg	pH of enzyme solution	Fixed enzyme		Substrate used
			μg	%	
Trypsin	2000 в 2 ml	7,9	1650	83	Wool
»	2180 » 4 »	7,9	1540	71	»
»	2000 » 4 »	3,9	1380	69	»
»	2000 » 4 »	7,9	1570	79	Silk fibroin
Chymotrypsin	2100 в 2 ml	7,9	1150	55	Wool
»	2000 » 4 »	7,9	1050	53	»
»	2000 » 4 »	3,9	1040	52	»
»	1550 » 4 »	7,9	850	55	Silk fibroin
Pepsin	4000 » 4 »	3,9	0	0	Wool
Papain	20000 » 4 »	3,5	0	0	Wool and silk fibroin

TABLE 2

Liberation of Protease Fixed by Wool and Silk Fibroin

Enzyme	Fixed enzyme		pH of enzyme solution	Washed out enzyme, μg	Liberated enzyme		Conditions of liberation
	μg	%			μg	%	
Trypsin	1400	70	7,9	42	130	10	8 *M* urea solution pH 7,1
»	1440	72	7,9	147	160	12	4 *M* urea solution pH 1,4
»	1630	82	7,9	300	430+ +80+ +30	40	2 *M* urea solution in 10% $CaCl_2$, 3 elutions
»	1220	61	7,9	172	840	80	8 *M* urea solution in 0,08*M* L-cysteine solution
»	1270	64	7,9	162	620	56	0,08 M L-cysteine solution
Trypsin*	1570	79	7,9	720	500	59	»
Trypsin in presence of 0.08 M L-cysteine	0	0	7,9	—	—	—	»
Chymotrypsin	1130	56	7,9	361	120	16	0,08 *M* L-cysteine solution
»	1050	53	7,9	156	120	13	8 *M* urea solution with 0,1*M* L-cysteine solution
»	1040	52	3,9	200	210	25	2%-$CaCl_2$ solution
»	800	40	3 9	300	240	48	2 *M* NaCl solution
»	1500	75	7,9	136	300	22	4 *M* urea in 2 M NaCl solution
»	1200	60	3,9	190	130	13	0,1 *M* L-histidine solution
»	1230	62	3,9	220	600	60	0,1 *M* L-histidine solution in 2*M* NaCl solution
Chymotrypsin with 0,1 M solution of L-histidine in 2 NaCl	0	0	7,9	—	—	—	—

* The experiment was carried out with silk fibroin; in the other cases, with wool.

We tried to liberate the fixed enzymes with acid buffer solutions, urea solutions, calcium and sodium chloride, cysteine, and histidine. The results are given in Table 2, from which we see that trypsin is best liberated by washing wool with 0.08 M solutions of L-cysteine, while the solutions of the other substances which we used for this purpose were much less effective. The presence of 0.08 M L-cysteine in the trypsin solution completely prevents fixation of the enzyme by wool.

Chymotrypsin is liberated from the fixed state on wool most effectively with 0.1 M L-histidine in 2 M sodium chloride solution. In this case, the presence of L-histidine and NaCl, just as the presence of L-cysteine in the experiments with trypsin, hinder the fixation of chymotrypsin on wool (Table 2).

We know from the literature that cysteine and some other compounds which contain the sulfhydryl group have an inactivating action on trypsin and chymotrypsin [7]. The idea has been expressed that in this case the inactivation is connected with reduction of the disulfide bond. It has also been shown that inactivation of trypsin by reducing substances in the presence of 8 M urea occurs much faster and at lower concentrations of reducing substance [8].

TABLE 3

Effect of 0.08 M L-cysteine* on the Autolysis of 0.05% Trypsin and Chymotrypsin Solutions (pH 7.9, 35°)

Time of incubation	Amount of enzyme by splitting casein, μg		Optical density of centrifugate after precipitation by TCA at 280 mμ	
	Trypsin	Chymotrypsin	Trypsin	Chymotrypsin
0	20	20	0,080	0,070
5	11,0	7	0,250	0,250
15	5,8	6,4	0,310	0,300
60	1,3	2,5	0,375	0,370
120	0,8	0,8	0,400	0,450

* Before determination of the enzyme by splitting casein, the enzyme solution was diluted 25 times with 0.0025 N HCl.

We studied the effect of L-cysteine on the hydrolysis of serum albumin by trypsin. We showed that L-cysteine at a concentration of 0.05 M does not inhibit the hydrolysis of serum albumin by trypsin.

Trypsin and chymotrypsin were submitted to autolysis in alkaline buffer solution with pH about 8 at temperature > 0° [9]. This process is accompanied by the appearance of peptides which are not precipitated by trichloroacetic acid, with simultaneous proportional lowering of the concentration of the enzyme protein and also decrease in its activity. The inactivation of trypsin and chymotrypsin prevents their self digestion, which we also used for determination of the effect of cysteine in the study of these enzymes. However, we found no inhibiting action of L-cysteine on the autolysis of trypsin and chymotrypsin (Table 3).

SUMMARY

Minced wool and silk fibroin fix trypsin and chymotrypsin from solutions with pH 7.9 and 3.9 at a ratio 150:1.

Trypsin fixed by wool or silk fibroin is liberated by washing with 0.08 M L-cysteine solution; chymotrypsin is liberated by 0.1 M L-histidine in 2 M NaCl solution.

L-Cysteine at concentration of 0.08 M does not inactivate trypsin and chymotrypsin.

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THE PROBLEM OF THE BIOCHEMISTRY OF THE THYMUS GLAND

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The experimental data which have been published up to now on the biochemistry and physiology of the thymus gland have not yet clarified fully the problem of the gland.

As early as 1929, Asher [1] obtained a preparation from the thymus, "thymocresin", which affected the growth of organisms; Bomskov [2] isolated a thermostable, water-soluble hormone from this gland; Bezsonoff and Comsa [3] prepared a pure extract. In the Bucharest Institute of Endocrinology, a protein extract of the thymus was prepared which had a many-sided action: anticarcinogenic, hypercalcemic, stimulating the growth of organisms, and a varied effect on metabolism. In recent years much work has been published to explain the composition of the thymus [4-8]; thus the isolation from it of thymohistones and nucleic acids has been reported.

Biological methods for determination of the thymus hormone are very limited. Among the recently developed methods we must mention that of Comsa [9], based on the antithyroid effect of the thymus gland.

In the published work from our laboratory [10-12], we have shown the protective action of the thymus gland in experimental cancer, which was studied even in 1935 by Parhon and Milcu [13].

We can conclude from our results that under the influence of the thymus there is a structural change in tumors and a suppression of their development with full disappearance, accompanied by a decrease in intensity of synthesis of phosphorus compounds, whose metabolism is strongly increased in neoplasms.

In the work reported at the Paris Conference in 1957 on radioisotopes, Parhon and Potop [14] described the effect of the thymus gland on the uptake of P^{32} in the organism of rats with tumors. It was there shown that the thymus gland had an effect on the rate of renewal of different phosphorus compounds, both in tumors and in various animal organs.

In a number of experiments carried out in our laboratory [15] it was shown that the thymus acted on phosphorus-calcium metabolism and on ossification, which indicates the existence of a biochemical relation between the thymus and the parathyroid gland. It was also shown [16] that the thymus affects the content of mineral substances in the endocrine glands. It was established, too, that the thymus increases the ability to bind water and affects phosphorus metabolism in the brain [17], stimulates the growth of organisms [18], and, evidently, stimulates the production of formed elements of the blood [19].

Starting from the results of our previous investigations and also considering the role of phosphorus in the physiology of the thymus and in the metabolism of the nucleotides, fats, and carbohydrates, we decided to investigate its phosphorus metabolism, and, in particular, to try to work out a biochemical test for thymus activity, based on changes in phosphorus metabolism. For this purpose we studied in this work the changes in different forms of phosphorus in the blood, liver, and adrenals in experimental removal of the thymus and after injection of its extracts.

In order to establish whether the thymus had a specific action, we carried out parallel experiments using protein extracts from muscle, prepared in the same way as the thymus extracts.

MATERIALS AND METHODS

The experiments were carried out on young and adult male rabbits and rats. The animals were separated into three groups. The first group consisted of animals in which the thymus gland was removed by operation; the second consisted of animals which were injected with 1 ml of thymus extract, corresponding to 0.5 g of fresh thymus per 100 g weight of the animal, daily for 21 days. The third group served as controls.

We studied the quantitative change in content of phosphorus compounds and the rate of inclusion of P^{32} in the blood, liver, and adrenal glands of all the groups of animals. We carried out separately the determination of total phosphorus (TP), acid-soluble phosphorus (ASP), nucleoprotein phosphorus (NPP), and lipid phosphorus (LP). At the same time we studied the change in body weight and amount of cholesterol in the adrenals.

TABLE 1

Change in Content of Phosphorus Compounds under the Influence of the Thymus Gland Calculated per 100 g of fresh tissue

Group of animals	No. of expt.	Fraction of phosphorus compounds				
		TP	ASP	NPP	LP	Calculated phosphorus
Rabbit						
Control	10	47 ±3,6	32,6±1,8	10,9±0,3	5,10±0,4	48,6
Thymus removed	10	48,5±2,7	30,7±0,6	16,7±0,7	2,47±0,6	49,1
Injection of thymus extract	10	73,9±2,7	40,1±0,7	25,2±0,4	7,6 ±0,4	73,5
Rat (adult)						
Control	20	29,0±1,2	12,6±0,5	12,1±0,4	2,8±0,2	27,5
Injection of thymus extract	20	45,6±1,2	18,6±0,2	20 ±0,5	5,3±0,1	43,9
Rat (not fully grown)						
Control	—	—	—	15,6±0,4	3,05±0,1	
Injection of thymus extract	—	—	—	24,5±0 8	2,3 ±0,2	

For the study of the rate of inclusion of P^{32}, the animals were injected intraperitoneally with a solution of $NaH_2P^{32}O_4$ with radioactivity corresponding to 20 μcurie per 100 g weight of the animal.

The radioactivity of the phosphorus compounds was determined with a Geiger-Muller counter and expressed either in counts or in μcuries. The results were calculated in terms of the standard error.

In some animals we studied the inclusion of P^{32} in the blood in vitro, by adding radioactive phosphate to the blood sample.

In a number of experiments, we studied the content of phosphorus compounds in the blood after injecting the animal over 21 days with 1 ml of muscle extract which contained 0.5 g of fresh muscle per 100 g weight of the animal.

RESULTS

Experimental athymia and hyperthymia cause changes in the content of phosphorus compounds in the blood (as Table 1 shows); athymia causes a lowering of rabbit LP by 106% and an increase of NPP by 57%. Injection of thymus extract causes an increase in amount of all fractions in the blood: in rabbits 131% for LP, 32.9% for NPP, 23% for ASP, and 35% for TP; in rats, 89% for LP, 64% for NPP, 47% for ASP, and 56% for TP.

In rats which were not fully grown, and in adult rats after injection of thymus extract, the amount of NPP increased by 65%. LP rose by 89% in fully grown rats and fell by 24% in young rats. As to the change in NPP we noted only a small difference because of age group.

TABLE 2

Effect of Thymus Gland on Inclusion of P^{32} in Phosphorus Compounds of Rabbit and Rat Blood
Counts per minute

Group of animals	No. of expt.	Phosphorus fraction				
		TP	ASP	NPP	LP	Calculated phosphorus
Rabbit						
Control	10	686±18	670±16	14±1,8	5,2±0,11	689,0
Thymus removed	10	616±29	596±18	15±2,1	4,3±0,21	615,8
Injection of thymus extract	10	725±25	687±20	36,2±2,8	6,60±0,20	728,
Rats						
Control	20	—	—	17,3±1,8	7,50±0,2	—
Injection of thymus extract	20	—	—	33,2±2,10	4,30±0,1	—

The data which we have given indicate the absence of a species difference in action of thymus extracts on the phosphorus compounds of the blood.

Special experiments showed the absence of any change in NPP and LP content in blood after injection of muscle extract, which indicates a specific effect of thymus extract.

The results given in Table 2 show that experimental athymia and hyperthymia cause changes in uptake of P^{32} by the blood. The data which we obtained show that under the influence of thymus extracts the P^{32} uptake is raised in the different forms of phosphorus compounds. Athymia produces decreased binding of P^{32}, especially in its lipid form, and also in the acid-soluble form. The results in athymia and after injection of thymus extract are opposite. Hence, the results obtained by using P^{32} are analogous to the results obtained by using chemical methods. In respect to the content of NPP and LP in rabbit and rat blood, we note no particular difference.

The thymus extract raises the uptake of P^{32} in the nucleoprotein and lipid fractions of blood phosphorus also, in vitro (Table 3). Evidently the effect of thymus on phosphorus metabolism in vivo and in vitro in blood is in the same direction. The difference is that in vivo this effect is shown more clearly.

In Table 4 we give data on the effect of the thymus on content of NPP and LP in rat liver.

It is clear from the table that under the influence of thymus extracts, the amount of NPP in the liver increases by 30.4% while the LP content remains almost unchanged. In experimental athymia the amount of NPP falls by 16.6% and the LP increases by 16%. Thus, the change in NPP is more evident in the liver, while the change in LP is more evident in blood.

The thymus extract stimulates uptake of P^{32} in different liver compounds. The greatest increase in P^{32} uptake is found in the ASP fraction (Table 5). These results parallel those obtained by the chemical method and show the active participation of the thymus gland in the phosphorus metabolism of the liver.

TABLE 3

Effect of Thymus Gland on Uptake of P^{32} in Blood in Vitro
Counts per minute

No. of expt.	Group	NPP	LP
8	Injection of thymus extract	150,0±3,4	64,0±0,8
9	Control	118,0±2,8	41,0±0,4

TABLE 4

Content of Phosphorus Compounds in Rat Liver after Removal of the Thymus Gland and When Its Extract Is Injected (in mg %)

No. of expt.	Group	NPP	LP
24	Control	148,0±2,80	2,94±0,30
12	Removal of thymus gland	124,0±2,08	3,42±0,14
12	Injection of thymus extract	193,0±2,44	2,30±0,22

TABLE 5

Inclusion of P^{32} in Phosphorus Compounds of the Liver after Injection of Thymus Extract
Activity in 10^{-6} μ curie

No. of expt.	Group	TP	ASP	NPP	LP	Calculated phosphorus
15	Control	0,67±0,06	0,13±0,04	0,25±0,04	0,32±0,03	0,70
15	Injection of thymus extract	1,33±0,04	0,56±0,07	0,34±0,01	0,43±0,02	1,33

In the adrenals, under the influence of athymia and injection of thymus extracts, the radioactivity changes as follows (for each group of animals we ran 10 experiments):

Experimental Conditions	No. of counts per min
Control	1542±28
Athymia	1840±30
Injection of thymus extract	1320±35

The P^{32} uptake in the adrenals rises in animals submitted to removal of the thymus and falls after injection of its extracts. These results parallel the change in weight of the adrenals; they are more obvious in animals with the thymus removed.

The cholesterol content rises in experimental athymia by 32% and falls by 32% after injection of thymus extract.

DISCUSSION OF RESULTS

These results show the possibility of using biochemical methods for studying the thymus gland with respect to its active effect on phosphorus metabolism.

It follows from these experiments that the changes in phosphorus compounds occur in opposite directions in athymia and after injection of thymus extracts; these changes are found in blood, liver, and adrenals.

From our earlier results and also from the experiments described here we can conclude that the thymus gland actively shares in phosphorus metabolism, serving, along with the parathyroid glands, as an organ which regulates phosphorus metabolism. According to Hammar [20] the relation between thymus and bone tissue is concerned with the content in it of phosphorus, nucleoproteins, and vitamins.

The results obtained in both types of animals (except for the greater content of ASP in rabbits) are almost the same. The effect of the thymus gland is related to an increased content of phosphorus compounds in blood and liver, especially NPP and LP.

The results obtained in parallel studies of P^{32} utilization by chemical determinations show that inclusion of P^{32} in phosphorus compounds after injection of thymus extracts also goes more intensely in blood and liver.

The inclusion of P^{32} in NPP and LP by blood in vitro under the influence of the thymus gland changes in the same direction as in the experiments in vivo. This shows the possibility of using P^{32} in vitro as a method for studying the function of the thymus gland.

In a study of the effect of irradiation on nucleoprotein metabolism, Stocken [21] observed a strengthening in specific activity of the thymus gland.

Considering the involution of the thymus when sexual maturity is reached, we studied this gland in immature and mature rats.

In rats of both ages, immature and mature, the action of the thymus was shown chiefly in respect to the lipid form of phosphorus, which increased in the blood of mature animals and fell in immature rats. It follows from this that the effect of thymus extracts on the synthesis of these compounds depends on age.

The values obtained in studying the effect of thymus extracts and athymia on liver phosphorus compounds show the increased content of phosphorus compounds (ASP, NPP, and LP) after injection of the animal with thymus extract, and the decrease in these compounds in athymia. LP increases in athymia and remains almost unchanged after injection of thymus extracts. These results show the stimulation of phosphorus metabolism in this organ.

The results obtained in experiments with P^{32} are analogous to the results obtained by chemical methods and show the active participation of the thymus gland in phosphorus metabolism of the liver.

We must emphasize that even now the determination of LP in blood and NPP in liver can be used to study thymus function.

The data on inclusion of P^{32} in the adrenals shows the strengthening of phosphorus metabolism in experimental athymia and its weakening when thymus extract is injected. Thus, the results described above, like the hyperplasia of the adrenals in athymia, show the possibility of working out a method for determining thymus activity based on the antagonism of these glands.

The interaction between the thymus and the adrenals has been shown by various authors. Weaber [22], for example, established the presence of specific destructive changes in the thymus gland after injection of cortisone and ACTH. Brolin and co-workers [23] showed the existence of an effect of the adrenal cortex on the involution of the thymus after injection of hexestrol.

In connection with this interaction of the glands, we have studied the changes of cholesterol in the adrenals and showed that its content is increased in athymia and lowered after injection of thymus extract, which confirms the results obtained by Comsa [24].

Thus, our data on the change in phosphorus metabolism in blood and liver by chemical methods and the method of P^{32} uptake indicate the possibility of biochemical estimation of the activity of the thymus gland. It is important to extend and deepen these investigations by a study of the phospholipid complexes, lipoproteins, and other phosphorus compounds of intermediate metabolism.

SUMMARY

Experimental athymia and the injection of thymus extract cause opposite changes in the content of some phosphorus compounds in the liver. The more constant changes are found in nucleoprotein and lipoprotein phosphorus. Changes in NPP are most clearly shown in the liver, and changes in LP, in the blood.

The data relating to binding of P^{32}, reinforced by the results of chemical determinations, show changes in phosphorus metabolism which have different directions in athymia and in injection of thymus extract.

The uptake of P^{32} in vitro gives results similar to the uptake in vivo, which indicates the possibility of using this method in the clinic.

Muscle extract does not show any effect on these phosphorus compounds, which indicates the specific activity of thymus extracts.

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THE AMINO ACID METABOLISM OF HEN EGGS

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In recent years conflicting data have been published in the Russian and foreign literature concerning the possibility of self renewal of proteins isolated from organisms (proteins of blood plasma, enzyme proteins, etc.), or proteins which have no cell structure such as the proteins of the yolk of hens' eggs and the proteins of the whites of fertilized and unfertilized eggs. Konikova and Kritsman and co-workers [1-3], using amino acids with labeled carbon, showed that glycine and some other amino acids could be taken up by isolated proteins. The authors consider this fact to indicate an autocatalytic process of self-renewal of proteins, that is, a very elementary form of protein metabolism. Sisakyan and Kuvaeva [4] in a study of metabolism in the cavity fluid (which lacks cell structure) of the mulberry silk worm during metamorphosis, observed the occurrence of regular enzymatic reactions which depended on self renewal and were the basis of the development which led to formation of cells and tissue structures.

On the contrary, Orekhovich, Levchuk, and Levant [5, 6] on the basis of their studies with labeled amino acids denied the possibility of synthetic processes and "renewal" of proteins in yolk and white of fertilized and unfertilized hens' eggs.

There is information in the literature concerning protein metabolism in developing hens' eggs. Various investigators [7-14] have shown that during incubation the nutritive substances of the yolk and white of the egg before they enter the vascular system of the embryo undergo a number of chemical changes. As a result, substances are formed which are used by the embryo as construction materials for tissues, as sources of energy, and for the synthesis of various biologically active substances. Needham and other authors [7, 8, 10] have confirmed that proteins are used least of all as sources of energy, and go almost completely (96%) into the tissues of the embryo. In this process, the yolk proteins and later the egg-white proteins are split hydrolytically to give, therefore, a "metabolic pool" of amino acids for the developing embryo.

The literature does not contain complete data on the "metabolic pool" of amino acids at different stages of development, but the pool should undergo changes with changing metabolic processes, processes of differentiation of organs and tissues, etc.

The possibility of regular change in the group of free amino acids depends on the presence and change in activity of the different enzymes of the hen egg. In the yolk and white of freshly incubated eggs, various enzymes have been detected [15], including proteolytic enzymes: peptidases, and proteases in the form of trypsin and erepsin.

The activity of these enzymes in the unincubated egg is very low, but as Engel'gardt and Vener [16], and also other authors [11, 17, 18] showed, with the beginning of development of the embryo it rises sharply and some enzymes which were absent earlier are formed at definite stages in the development of the egg.

Evidently, in different kinds of developing animals we can find analogies in the change of activity of enzymes and the qualitative and quantitative composition of the "metabolic pool" of amino acids.

Thus, Deuchar [19] observed considerable variation in eight different free amino acids at different stages in the development of Xenopus laevis and studied the formation and role of the different amino acids, chiefly aspartic and glutamic acids, in the processes of morphogenesis and the synthesis of proteins in the developing embryo. Kavanau [20] obtained analogous results in a study of amino acid metabolism in different stages in the development of the sea urchin. However, up to now there remain many questions in the study of protein and nitrogen metabolism of the embryo which require solution.

It is true that the literature contains very confused and unsystematic data on the change in chemical composition and on the presence of metabolic processes in the incubated, unfertilized egg.

These data have a definite interest in comparison with the studies of parthenogenetic development of sea urchin eggs, fish roe, and unfertilized hen eggs [21]. In the paper of Ferdinandov [22] are given data on the absorption of oxygen and the excretion of carbon dioxide by the yolks and whites of unfertilized eggs during incubation.

In the present investigation we wished to study the dynamics of the dicarboxylic amino acid content and the total amino acid nitrogen in the developing hen egg and also in the yolk and white of incubated unfertilized eggs.

EXPERIMENTAL METHODS

As the material for study we used eggs of White Leghorns, maintained on a constant and complete ration. Unfertilized eggs were obtained from hens isolated from cocks for many months, but kept under the same conditions as the ordinary flock. Incubation was carried out in a laboratory egg incubator with a water heater. The incubation regime was the one customary in hatcheries.

In the unfertilized eggs we determined the nitrogen of the dicarboxylic amino acids, the total amino acid nitrogen, and the dry residue, separately in yolks and whites for each 5 days of incubation. In the fertilized eggs we determined the same components separately in the white, the yolk, and the embryo. For this, on the day of the analysis the eggs were frozen, opened, and while frozen the embryo, yolk, and white were separated from the other fluids and tissues.

The nitrogen of the dicarboxylic amino acids was determined by the chromatographic method of Kretovich and Bundel' [23], the total amino acid nitrogen by the Folin method as modified by Vilenskii [24] with sodium 1,2-naphthoquinone-4-sulfonate.

RESULTS

The change in content of the dicarboxyl fraction of the amino acids during incubation is shown in Table 1, from which we see that the concentration of dicarboxylic amino acids in the yolk and white of the egg undergoes considerable variation. In the fertilized egg the change in concentration of dicarboxylic amino acids is more considerable than in the unfertilized egg.

TABLE 1

Content of Dicarboxylic Amino Acid Nitrogen in Incubated Fertilized and Unfertilized Hen Eggs (in mg%)

Day of incubation	Number of eggs studied	Yolk				White				Embryo
		Fertilized eggs		Unfertilized eggs		Fertilized eggs		Unfertilized eggs		
		By wet weight	By dry weight	By wet weight	By dry weight	By wet weight	By dry weight	By wet weight	By dry weight	By wet weight
Before incubation	7	22,5	46,4	22,5	46,4	24,0	200,1	24,0	200,1	—
5	8	18,9	42,8	15,8	32,8	38,0	203,2	36,5	266,4	18,4
10	5	13,5	32,3	20,6	45,5	66,9	182,3	18,5	208,0	27,6
15	5	18,9	40,5	13,5	30,0	57,7	146,8	36,4	251,0	37,6
20	10	32,5	75,5	16,5	39,0	—	—	35,3	195,0	30,3

The observed changes cannot be explained exclusively by a shift of water from the white to the yolk, but also by evaporation of water from the white, which, to judge from the data of Table 2, occurs in the incubation process, since when we calculate the concentration of dicarboxylic amino acids on the dry weight, the previous regularities in variation of their concentrations are preserved (Table 1).

In the yolk of fertilized eggs from the middle of the incubation period the concentration of dicarboxylic amino acids decreases by about 40% of the initial value. In the second half of development, their content in the yolk rises sharply, by about 150% compared to the unincubated eggs.

TABLE 2

Content of Water in White and Yolk of Incubated Fertilized and Unfertilized Hen Eggs (in mg%)

Day of incubation	Number of eggs studied	Yolk		White	
		Fertilized eggs	Unfertilized eggs	Fertilized eggs	Unfertilized eggs
Before incubation	5	51,6	51,6	88,1	88,1
5	6	55,8	51,8	81,3	86,3
10	5	58,3	55,2	63,3	86,3
15	3	53,4	54,9	60,7	85,3
20	4	57,0	57,7	—	81,9

In the whites of the fertilized eggs, the concentration of the dicarboxylic amino acids rises considerably, because of their loss of water. Their content on the dry weight decreases regularly and on the 15th day of development is only 70% of the starting value.

In the embryo the contents of dicarboxylic amino acids rises to the 15th day of development, and during the last five days their concentration decreases somewhat.

TABLE 3

$N-NH_2$ Content in Incubated Fertilized and Unfertilized Hen Eggs (in mg%)

Day of incubation	Number of eggs studied	Yolk				White				Embryo
		Fertilized eggs		Unfertilized eggs		Fertilized eggs		Unfertilized eggs		By wet weight
		By wet wt.	By dry wt.	By wet wt.	By dry wt.	By wet wt.	By dry wt.	By wet wt.	By dry wt.	
Before incubation	7	237,8	491,3	237,8	491,3	33,4	280,6	33,4	280,6	—
5	5	292,1	660,8	188,6	391,3	93,6	800,0	61,3	448,8	120,1
10	5	281,3	674,5	195,6	436,6	172,9	471,1	42.1	307,3	105,9
15	5	362.1	777,0	180,2	400,0	180,7	459,8	44,6	307,5	127,7
20	10	287,0	667,4	171,2	404,7	—	—	65,6	362,4	149,3

In the white and yolk of unfertilized eggs the content of dicarboxylic amino acids (calculated on the dry substance), considering the accuracy of the analysis, was the same at the end of incubation as in the unincubated eggs.

Their changes during incubation showed some interesting regularities (Table 1): on the fifth day of incubation, the dicarboxylic amino acid content in the yolks decreased by 30%, and in the whites, at the same time, it increased by 33%. On the 10th day of incubation there was a reversed shift of the dicarboxylic amino acids from the white into the yolk. On the 15th day of incubation the dicarboxylic amino acids again were transferred from the yolk into the white, and on the 20th day, again into the yolk.

In the yolks of fertilized eggs, the total amino acid nitrogen content ($N{-}NH_2$), as Table 3 shows, gradually increased to the 15th day of development and then decreased somewhat. However, this decrease occurs only in the monocarboxyl fraction. This is clear when we compare the data of Tables 1 and 3; during the last five days of incubation the content of dicarboxylic amino acids in the yolk increases almost three times.

In the whites of fertilized eggs on the fifth day of development, the content of $N{-}NH_2$ rises almost three times.

Then the amino acid concentration continues to increase because of removal of water from the white, but when calculated on dry weight, the total amount of amino acid nitrogen in the whites falls sharply, parallel with the increased content of it in the yolks.

TABLE 4

Content of Dicarboxylic Amino Acid Nitrogen and Total Amino Acid Nitrogen in Unfertilized Eggs at Different Periods of Incubation

Day of incubation	Number of eggs studied	Average wt. of eggs	Dicarboxylic amino acid N		Total amino acid N	
			mg	mg%	mg	mg%
Before incubation	7	34,5	8,56	24,8	271,2	785,2
5	5	41,4	12,0	28,9	220,6	532,1
10	5	33,6	9,31	27,7	237,7	709,5
15	4	39,4	11,23	28,5	224,5	571,2
20	6	35,3	9,62	27,2	240,1	680,1

In the embryo the total amino acids regularly increase during the whole period of development, with small and brief decreases in the middle of the incubation.

In the unfertilized eggs we find the same regularities in shift of $N{-}NH_2$ from the white to the yolk and reverse as for the dicarboxylic amino acids: after five days the $N{-}NH_2$ content in the yolk falls by 20% and in the white rises by 60%. After 10 days of incubation the $N{-}NH_2$ content in the yolk rises by 10% and in the white, falls by 31%.

Thus, here we do not find a proportional decrease and increase of $N{-}NH_2$ in yolk and white, and in a number of cases this difference is very considerable.

At the end of the incubation period, that is, after 20 days, we find a decrease of about 17% in the $N{-}NH_2$ content of the unfertilized egg yolk, and a 29% rise in its content in the white.

Our results permit us to conclude that there is a constant content of dicarboxyl fraction nitrogen and total amino acid nitrogen in the whole unfertilized egg during the incubation period.

To test this assumption, we carried out a series of determinations of the above-mentioned nitrogen fractions at different periods of incubation in the whole unfertilized egg. Parallel with this we determined the content of water in the yolks and whites (Table 4).

Table 4 gives the figures for content of dicarboxylic amino acid nitrogen and total amino acid nitrogen in the whole egg for each five days of incubation. As is clear from the table, during the 20 days of incubation the variation in content of dicarboxylic amino acid lies within the limits of accuracy of the analysis. The small increase in concentration to the 20th day, about 10% compared to the unincubated egg, can be explained by the disappearance of water.

In our previous work we showed [25] that unfertilized eggs during incubation lose 10-11% water as the result of evaporation.

The amount of total $N-NH_2$ in unfertilized eggs remains practically unchanged at the end of 20 days incubation (Table 4). On the fifth day of incubation there is a short decrease in $N-NH_2$ content of about 30%.

DISCUSSION OF RESULTS

Our results on the variation in content of dicarboxylic amino acids and total amino acids in the developing egg of the hen confirm the data obtained previously by many investigators and reflect the general biological regularities of amino acid metabolism in developing eggs of the chicken and other animals.

We believe that the data obtained in the incubation of unfertilized eggs is especially interesting. We carried out these experiments in order to fix the limits of biochemical transformations in the yolk and white, which are connected with the development of the embryo and the chemical and physicochemical processes which could proceed in the egg independent of the development of the embryo.

The data of the authors whom we cited [21, 22] on the occurrence of glycolytic processes, absorption of oxygen, formation of carbon dioxide, and variation in temperature in the incubation of unfertilized eggs were still not sufficiently convincing to show the existence of processes of metabolism in the unfertilized egg.

The rather considerable variation in concentration of dicarboxylic amino acids and total amino acids which we observed in yolk and white of the unfertilized egg (calculated on wet substance) during the incubation can be partly explained by shift of water between yolk and white. Water metabolism in the developing fertilized egg has been rather fully studied. Shift of water has been described from the white into the yolk at definite stages of development, and then transfer of water into the amnion and allantois; the evaporation of water and its absorption by the embryo have been studied at different stages of development, etc. The water metabolism of the unfertilized egg has been less studied and we have found no information in the literature on results which interest us.

The water shifts which we have observed from the white into the yolk in the same period and in about the same quantitative ratio as for the fertilized egg, allows us to seek an explanation for this phenomenon, not in the processes of metabolism, but in the changes in physicochemical properties of the egg. For example, we can assume a disturbance in osmotic equilibrium between yolk and white which strengthens the dissociation of electrolytes under the influence of the incubation temperature. We can also see the reason for this in the change in permeability of the yolk membrane under the influence of the incubation conditions, and also the change in physical and chemical properties of the yolk and white. Since we do not at present have sufficient experimental evidence as to these assumptions, we can only confirm that the shift of water between white and yolk in both fertilized and unfertilized eggs, especially in the first half of the incubation period, is some sort of chemical or physicochemical process, independent of the developing embryo.

When we calculate the content of the amino acids which we studied on dry weight, their variation during incubation is much decreased, but it still remains considerable. As we observed earlier, the increased content of amino acids in the yolk is related to a decrease in their content in the white, and vice versa.

Determination of the gross content of dicarboxylic amino acids and total amino acid composition allows us to conclude that processes of splitting proteins do not occur in the unfertilized, incubated egg.

As Table 4 shows, the content of nitrogen from dicarboxylic amino acids and total amino acids in the whole unfertilized egg is almost unchanged during the 20 days of incubation.

The slight increase in content of dicarboxylic amino acids on the 20th day is quantitatively related to loss of water by the egg in the incubator.

SUMMARY

In the yolks and whites of fertilized eggs at different stages of incubation there is considerable variation in content of the nitrogen of dicarboxylic amino acids and in total amino acid nitrogen. This indicates proteolytic splitting of the proteins in the egg and the use of the free amino acids by the developing embryo.

In the whole unfertilized egg during the 20 days of incubation, the content of nitrogen of dicarboxylic amino acids and total amino acid nitrogen remains almost unchanged, due to the absence of processes of synthesis and splitting of proteins.

In the incubated unfertilized egg we have observed a shift of water from the white to the yolk of about the same magnitude and at the same period as in the fertilized egg.

In the unfertilized egg we find a shift of amino acids at different periods of incubation from the white to the yolk and the reverse, evidently as a result of disturbance of osmotic equilibrium between white and yolk, and also a change in permeability of yolk membrane when the egg is incubated.

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INCREASING THE VITAMIN P CONTENT AND IMPROVING THE QUALITY OF TEA

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The studies of Kursanov, Bukin, and co-workers [1-3] showed that tea tannins have strong vitamin P properties and increase the resistance of the blood-capillary walls. Tea tannins surpass considerably in vitamin P activity such substances as citrin from citrus fruits and rutin from buckwheat flowers. In view of these studies, it is very important to seek a process which will permit raising the tannin content in tea and thus increasing its physiological value.

In the production of black tea by the present process of treating tea leaves, during fermentation there is a deep oxidation, condensation, and precipitation of tea tannins. As a result, in commercial tea only about 50% of the tannins of the original source remain in solution. This occurs because the fermentation process, particularly in its second phase, produces not only oxidation of the tannins, but also their precipitation by proteins. The oxidation of tannins begins even in the first phase of fermentation, during the twisting process, but it is greatly strengthened and accompanied by precipitation of tannins in the second phase of fermentation.

If the fermenting action is stopped by high temperature at the end of the first phase, and the second phase, and the second phase of fermentation is replaced by thermal treatment, we obtain a tea with a high content of soluble tannins associated with a pleasant taste and aromatic qualities. Such a possibility of regulating the biochemical processes gives a new technological process for black-tea production, which has been worked out in the A. N. Bakh Institute of Biochemistry of the Academy of Sciences, USSR, and which permits an increase in the tannin content in tea and actually improves its quality [4, 5].

Experiments which we carried out under production conditions in 1957 in the Georgian SSR, and also in 1958 in the Georgian SSR, Azerbaidzhan SSR, and in the Krasnodar region showed that the new technology makes it possible to raise the tannin content of tea by 3-6%, and thus to improve its taste and aroma. This is clearly shown in Table 1 which gives the results of determination of the soluble tannins in samples obtained by the new technology (experimental) and the present method (control) in different tea factories in the 1958 season.

As the data of Table 1 show, all the experimental samples, prepared by the new method, have an increased tannin content compared to the controls prepared by the existing process.

Besides the determination of the soluble tannins by the method of Loewenthal, we isolated the ethyl acetate fraction of the tannins after treating the tea in a Soxhlet apparatus with a mixture of chloroform and benzene.

These data are given in Table 2.

The data given in Table 2 show that from 30 g of tea obtained by the present process we could isolate 3.1 g of ethyl acetate fraction of tannins, which is 10.3% of the dry weight, while from 30 g of tea prepared by the new process, the yield was 5.5 g or 18.3% of the dry weight. This indicates that the new process gives an additional 80 g of tannins, that is, vitamin P, per kilogram of tea. Thus, if the whole tea industry should use the new method, supplying the nation with tea of high quality, it would supply 80 g of vitamin P for every kilogram of tea.

TABLE 1

Content of Soluble Tannins in Samples Obtained by the New Process (Experimental) and the the Present Process (Control) in the 1958 Season in Different Tea Factories

Name of factory	Manufacture of tea, 1958	Fraction	Tannin content*		Increase in tannin over present process
			Present process (control)	New process (experimental)	
Anaseulsk Tea Factory, Georgian SSR	July	I+II	14,8	19,3	+4.5
		III	11,1	16,3	+5,2
Dagomyssk Tea Factory, Krasnodar region	August	I	12,4	15,4	+3,0
		III	11,8	14,3	+2,5
Astarinsk Tea Factory Azerbaidzhan SSR	September	I+II	10,7	12,1	+1,4
		III	9,9	13,1	+3,2

* Tannins determined according to Loewenthal.

Catechin Content of New-Process Tea

Besides determination of the tannins according to Loewenthal and preparative separation of the ethyl acetate fraction, we were also interested in the catechin composition of the tea tannins from the new process, since according to the literature [6,7] in the present type of tea only traces of catechins are present. We therefore determined the catechins by the method of paper chromatography [8].

TABLE 2

Yield of Ethyl Acetate Fraction of Tea Tannins from New and Present Processes

Type	Amount of tea used, g	Yield of ethyl acetate fraction		Difference	
		g	%	g	%
Present process (control)	30	3.1	100	–	–
New process (experimental)	30	5.5	145	+2.4	+45

The resulting chromatogram is given in Fig. 1. As Fig. 1 shows, for the new-process tea, six catechins are characteristically present: L-epigallocatechin, L-epicatechin + DL-catechin, DL-gallocatechin, L-epigallocatechin gallate, L-epicatechin gallate. In the tea prepared by the old process we find only 3 catechins: L-epicatechin gallate, L-epigallocatechin gallate, and L-epicatechin + DL-catechin. These catechins are also present only in small amounts. Analogous data which indicate that in tea obtained by the usual method only traces of catechins are present have been presented in the work of Zaprometov and Soboleva [6] and of Dzhemukhadze and Shal'neva [7].

A clearer picture to confirm the difference in quantitative composition of catechins in tea from the new and present processes is obtained by densitometric measurement of the intensity of the colored band produced in the Shipalov photoelectric densitometer [8]. The results given in Fig. 2 confirm the considerably greater content of catechins in the new process tea.

Thus, the results of this investigation show that the new process not only increases the tannin content of black tea, but also retains the catechins in greater amounts. This fact indicates that the new process gives a more valuable black tea from the physiological viewpoint.

Organoleptic Analysis of Tea From The New And Present Processes

The new process not only increases the tannin content in commercial tea, but also considerably improves the taste and aromatic properties of the product. In order to show how the quality of the tea is improved in the new process, tea samples obtained by the new and present processes were submitted to organoleptic analysis.

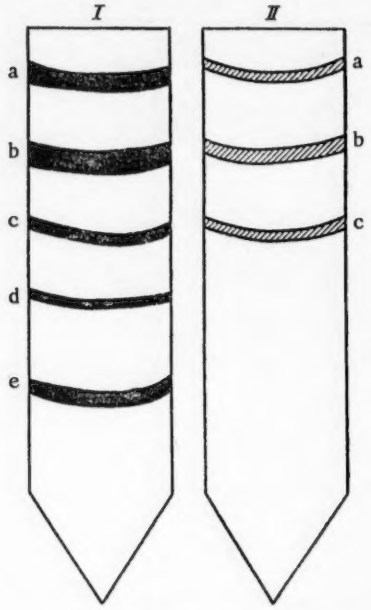

Fig. 1. Chromatogram of tea catechins from tea of the new and present processes: I) new-process tea (experimental); II) present-process tea (control). a) L-epicatechin gallate; b) L-epigallocatechin gallate; c) L-epicatechin + DL-catechin; d) DL-gallocatechin; e) L-epigallocatechin.

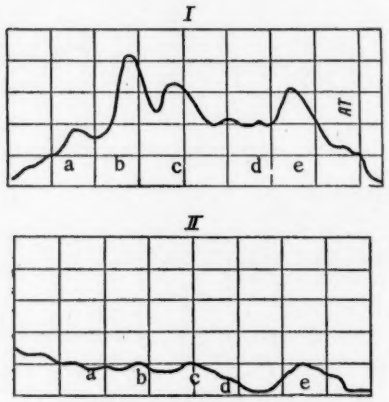

Fig. 2. Absorption curves of catechins in tea from the new and present processes: I) new-process tea (experimental); II) present-process tea (control). a) L-epicatechin gallate; b) L-epigallocatechin gallate; c) L-epicatechin + DL-catechin; d) DL-gallocatechin; e) L-epigallocatechin.

In Table 3 we give data on the testing of experimental and control samples of tea obtained from the Anaseulsk, Dagomyssk, and Astarinsk Tea Factories.*

TABLE 3

Results of Tea-Tasting Tests of Samples Obtained by the New and Present Processes in the 1958 Season from Different Tea Factories

Name of factory	Treatment of tea, 1958	Fraction	Present process (control)		New process (experimental)		Difference	
			Aroma	Taste	Aroma	Taste	Aroma	Taste
Anaseulsk Tea Factory, Georgian SSR	July	I+II	2,5	2,75	2,75	3,25	+0,25	+0,5
		III	2,0	2,0	2,25	2,25	+0,25	+0,25
Dagomyssk Tea Factory, Krasnodar region	September	I	1,75	2,0	2,5	2,5	+0,75	+0,5
		II	1,5	1,25	2,0	2,25	+0,5	+1,0
Astarinsk Tea Factory Azerbaidzhan SSR	September	I+II	2,5	2,75	3,0	3,5	+0,5	+0,75
		III	2,0	1,75	2,25	2,5	+0,25	+0,75

*The tests were carried out by tea-taster A. I. Grigor'ev.

As Table 3 shows, all the experimental forms surpassed the controls in quality (aroma and taste) by 0.25-0.75, and in some samples, by 1 point.

It is known that increasing the quality of tea by 0.1 point gives the industry about 40 million rubles of additional income. This means that if the tea industry of the USSR should use the new process, it would give the state an additional income of 200 million rubles per year because of the improved quality of the product.

SUMMARY

A study of the content of soluble tannins in tea from the new and present processes of manufacture has shown that in the new-process tea the content of soluble tannins is 2-5% greater than in the present-process tea. From 30 g of the new tea we have isolated an ethyl acetate fraction of 5.5 g or 18.3% of the dry weight, while from the same quantity of the present tea we have isolated only 3.1 g or 10.3% of the dry weight. This means that the new tea gives 80 g of additional tannin, that is, vitamin P, per kilogram.

A study of the catechins by the method of paper chromatography has shown that the new tea contains six different catechins, while in the present tea there are only traces of three catechins.

Organoleptic analysis has shown that the new tea is 0.25, 0.5, 0.75, and in some cases 1.0 point, better in aroma and taste than the present tea. The increased quality of tea of 0.1 point gives the industry about 40 million rubles of additional income. This means that if the tea industry of the USSR should use the new process, it would give the state an additional income of a minimum of 200 million rubles per year due to the improved quality of the product. Hence, it is important to introduce the new process widely into production. GNTK of the Azerbaidzhan SSR has already decided on wide introduction of the new process in the production of black tea. It is desirable that this example be followed in other tea regions of the USSR.

The introduction of the new process will raise considerably the vitamin P properties of the tea and thus supply a product of high quality and physiological value.

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SCIENTIFIC INFORMATION

BIOCHEMISTRY IN POLAND

After the establishment of popular power in Poland, favorable conditions were set up for the development of all branches of science and for the formation of groups of young scientists who worked energetically to develop Polish biochemistry.

Scientific research and teaching in biochemistry developed in Poland both in special institutes and in departments of the higher educational institutions.

The Institute of Biochemistry and Biophysics, as part of the Polish Academy of Sciences, is now in a period of growth. This Institute, directed by Prof. J. Heller, consists of several laboratories which, until the construction of the new buildings of the Institute, are located chiefly in quarters in Warsaw University. Work is carried on in the Institute on the biochemistry of insects, enzymology, the biochemistry of microorganisms, the biochemistry of plants, and on comparative biochemistry.

Another center of biochemistry is directed by Professor W. Nemerko, the Division of Biochemistry in the M. W. Nencki Institute of Experimental Biology. In the laboratories of this Institute, work is carried out on the biochemistry of insects; particular attention is paid to the study of metabolism of phosphorus compounds and lipids in the metamorphosis of insects.

Centers for scientific research and teaching in the field of biochemistry are also found in the medical institutes located in the major cities of Poland. Among these centers, for instance, is the Department of Physiological Chemistry of the Krakow Medical Academy, directed by Prof. B. Skarzynski. In this department work has been carried on for many years by the famous biochemist, Prof. L. Marklewski, whose name is widely known as one of the basic workers in the field of the chemistry of chlorophyll. At the present time the construction of a new building, especially designed for the Institute of Physiological Chemistry, is under way. In the laboratory of this Institute investigations are carried out on the biochemistry of vitamin B_{12}, the biochemistry of autotrophic bacteria, and work on the use in biochemical investigations of the special enzymatic micromethods developed in the Carlsberg laboratories by K. Linderstrom-Lang and H. Holter.

When we speak of departments of biochemistry in medical institutes, we must mention the department in the Medical Academy of Wroclaw under the direction of Prof. T. Baranowski. This department is located in the old building of Wroclaw University. The chief work in this laboratory is the isolation of crystalline proteins and enzymes. Prof. T. Baranowski is also the director of the biochemical division of the Hirschfeld Wroclaw Immunological Institute, where investigations are carried out on the isolation, purification and study of protein fractions from blood serum and on the investigation of immunochemical reactions.

One of the centers of biochemical investigation is the Department of Biochemistry of the Medical Academy in Lublin, whose director is Ya. Open'skaya-Blyaut.

Here the studies are chiefly on developing, perfecting, and applying the methods of chromatography in biochemistry.

There are also actively working departments of biochemistry in all the other Medical Academies (Institutes) in Gdansk, Poznan, Lodz, and other cities.

Divisions of biochemistry and biochemical laboratories have been organized in a number of branch institutes. Thus, for example, a well-equipped division of biochemistry exists in the State Institute of Hygiene, an old research organization in which, in his day, worked one of the founders of vitaminology, C. Funk. In the laboratory of this institute, investigations are carried on in the field of nucleotides and nucleic acids by spectrophotometric methods, and also work on the biochemistry of vitamins and on biochemical investigation of different food products.

A biochemical division has been formed in the recently built Skierniewice Horticultural Institute. In this division they investigate the development of methods for determination and study of the transformations of ascorbic acid and vitamin P.

Studies in the field of technical biochemistry are carried out in the biochemical division of the State Institute of Cereal Culture in Warsaw, in the biochemical laboratory of the State Fermentation Institute, and in a number of branch institutes.

Studies on the biochemistry of plants are developing in the biochemistry department of the Warsaw Agricultural Institute. This department is directed by Prof. I. Reifer. Here studies are carried out on the biochemistry of the formation of alkaloids in plants, on the effect of some stimulators of metabolism and growth of plants, and on reactions of the ornithine cycle in plants.

In the Poznan Agricultural Institute under the direction of Prof. J. Janicki, work is carried out on the biochemistry of grain and on the biosynthesis of vitamin B_{12} in propionic bacteria.

The coordination of all the activities of Polish scholars in the field of biochemistry is carried out by the Biochemical Committee established by the Polish Academy of Sciences. Under the protection of this committee the publication of two special biochemical journals has been organized. One of these, Acta Biochemica Polonica, is a medium of publication for original work in biochemistry. This journal is issued in the Polish language and each paper has a summary in three languages, including Russian. The second journal, Progress in Biochemistry, appears quarterly and publishes reviews of important problems in biochemistry, translations of reports and lectures of eminent biochemists, information on congresses, colloquia and meetings, as well as material which illustrates the state of biochemistry in different countries and large institutions. This publication is very useful for young workers and raises the scientific level of Polish biochemists. Recently, the journal Progress in Biochemistry has published, for example, translations of the Nobel Address of G. Theorell, a review by D. Shugar on spectrophotometric investigation of nucleotides and nucleic acids, a series of papers on the biochemistry of plants, special papers which discuss the present state of the biochemistry of photosynthesis, papers on the work of such great centers of biochemical activity as the Carlsberg laboratory in Copenhagen, and papers on the biochemistry and physiology of gibberellin.

Along with the publication of original experimental work in the journal Acta Biochemical Polonica, Polish biochemists also issue monographs and scholarly literature. Here it is especially important to note the very valuable collection of materials on the life and activities of M. W. Nenck [1]. In this collection are a series of new documents and materials on this great scientist, one of the founders of modern biochemistry. The collection contains a complete biography of M. Nencki and his co-workers, a series of letters from N. Nencki to L. Marklewski, papers of Oscar Low, M. Hahn, and V. Zav'yalov, written in memory of M. Nencki, and also the classical work of M. Nencki himself, "The Biological Relations of Leaf Pigments and Blood Pigments", and his famous speech at the meeting of Polish Natural Scientists and Physicians in Krakow in 1900, "The Problems of Biological Chemistry."

There is great interest in the volume which Prof. B. Skarzynski has prepared for the press [2] of the collected scientific and public works of Andrei Sniadecki, the famous Polish naturalist and author of "Theory of Organic Nature." This work, first published in 1804, and then translated into a number of European languages, played in its time an important part in the development of physiology and in the foundation of the idea of the importance of metabolism in life processes.

A group of Polish biochemists and chemists under the leadership of Prof. Ya. Open'skaya-Blyaut have published an important handbook on chromatographic analysis [3], in which there is a detailed and documented treatment of the literature on present methods and modifications of chromatographic analysis. This work will certainly permit wide distribution and development of chromatography in scientific research carried out in Poland.

Polish biochemists also work on the creation of textbook literature. Among the publications of this nature, we must mention the short Course in Physiological Chemistry of Prof. T. Baranowski, published by the State Medical Press of Poland [4], the 1st volume of a Course in Physiological Chemistry of Prof. B. Skarzynski, published by the State Agricultural Press in Warsaw [5], and the excellent practical handbook on the biochemistry of plants by W. Brzeski and Z. Kaniuga, young workers in the Department of Biochemistry of the Higher Agricultural institute in Warsaw [6].

We must also mention the fact that Polish biochemists have translated into Polish a number of monographs, textbooks, and practical handbooks on biochemistry. Thus, for example, they have translated and published: the monograph of A. Chibnall on the nitrogen metabolism of plants, the summary of D. N. Pryanishnikov "Nitrogen in the Life of Plants and in the Farms of the USSR", the "Practical Handbook of Animal Biochemistry" of S. E. Severin and N. P. Meshkova, "Practical Handbook of Plant Biochemistry" of A. N. Belozerskii and N. I. Proskuryakov, "Enzyme Activity of Protoplasmic Structures" of N. M. Sisakyan, "Basic Plant Biochemistry" of V. L. Kretovich, "Biochemistry of Grain and Products of Its Treatment" of N. P. Koz'mina and V. L. Kretovich, and a number of other publications. At the present time they are translating the latest edition of the book of A. I. Oparin, "The Origin of Life on Earth" and the "Basic Dynamics of Biochemistry" edited by E. Bolduin.

It is very satisfactory to see that in the Polish biochemical laboratories, the older generation has gathered an energetic and able group of young scholars who, in the new Poland are establishing conditions for the development of their enthusiasm and ability.

V. L. Kretovich

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CONFERENCE ON " PHOSPHORYLATION AND FUNCTION"

(Leningrad, June 13-18, 1958)

When, a half century ago, Ivanov and Garden and Young observed phosphorylation of carbohydrates in alcoholic fermentation it was difficult to believe that this phenomenon would be related in any way to oxidation processes. The understanding of this relation, now evident to everyone, was attained later, in the thirties, when the interrelation of phosphorylation and oxidation was traced in the aerobic and anaerobic metabolism of cells. Somewhat later, the immense biological significance of oxidative phosphorylation became clear. It initiated the numerous processes of phosphate transfer which occur at different energy levels between substances which belong to many classes of organic compounds during different biochemical reactions. Phosphate transfer, which consists in combination by phosphorylation and dephosphorylation of organic compounds, emerges as one of the most universal and characteristic features of metabolism. Biosynthesis, various forms of mechanical motion, secretion, osmotic activity, production of electrical impulses and radiant energy, cell division, all these varied phenomena of life are carried out by the energy of macroenergetic bonds of the adenylic system which is formed as a result of oxidative phosphorylation.

It is not strange that this process has attacted the attention not only of biochemists, but also of physiologists, pharmacologists, and pathologists who seek to find here the solution of the problems which concern them. However, it is strange that until now oxidative phosphorylation has never been the theme of any special conference or symposium.

The Institute of Experimental Medicine of the Academy of Medical Sciences, USSR, became the pioneer in this respect when it initiated the first conference on oxidative phosphorylation under the somewhat more general title "Phosphorylation and Function", held in Leningrad between the 13th and 18th of June, 1958. The organizing committee (chairman, Corresponding Member AMN SSSR Prof. V. S. Il'in, members, Prof. N. A. Kharauzov, Prof. S. A. Neifakh, E. P. Zdrodovskaya, and K. G. Gromova) and the members of the Division of Biochemistry of the IÉM* carried on the heavy work of preparing the conference. The participants comprised biochemists from Moscow (130 persons), Leningrad (180), Kiev (8), and other cities; in all 350 participants. Forty-one papers were heard, including 8 from the Division of Biochemistry of the IÉM, 4 from the laboratory of biochemistry of animal cells in the Institute of Biochemistry, Academy of Sciences, USSR, 3 from the department of biochemistry of the S. M. Kirov Military Medical Academy, 2 from the Department of Animal Biochemistry, Moscow State University, etc.

In his introductory words, the Chairman of the Organizing Committee, Prof. V. S. Il'in, noted the great significance of the study of oxidative phosphorylation in modern biochemical ideas and emphasized that some 25 years ago the investigations of V. A. Éngel'gardt were published, showing experimentally, for the first time, the existence of oxidative phosphorylation at the cellular level. This work was the subject of the paper of A. A. Baev, "Development of the Study of Oxidative Phosphorylation (25 Years after Its Discovery)" in which he gave a survey of the further studies in this field to about 1945. The conference listened with great attention to the paper of T. V. Venkstern, A.A. Baev, and V. A. Éngel'gardt, "Transformations of Nucleotides in Nucleated Erythrocytes in Relation to Respiration."

The authors set themselves the problem of repeating the experiments of V. A. Éngel'gardt on oxidative phosphorylation which had been carried out in 1930-1932 on pigeon erythrocytes, but with the use of modern analytical methods (chiefly paper chromatography). As a result, they identified products of anaerobic splitting of nucleotides and their aerobic resynthesis in the erythrocytes (this was not feasible in 1930-1932), and they

* Institute of Evolutionary Morphology.

showed that under the experimental conditions there was true oxidative phosphorylation (combined with respiratory binding of inorganic phosphate, phosphorylation of ADP and synthesis of ATP). Thus, this study fully confirmed the results of 1930-1932.

In the review paper of V. P. Skulachev, "Phosphorylative and Respiratory Chains" he discussed the idea that the low value of the phosphorylation coefficient (mole ratio of bound P to absorbed O_2) which is usually found in intact tissues and under definite conditions in experiments with mitochondria and enzyme systems, does not depend on activation of the phosphatase reactions and injury to the mechanisms of combination, but is the result of a particular chain of oxidation-reduction reactions which proceeds independently of phosphorylation. The paper caused a lively exchange of opinions.

In connection with improving the accuracy of the value of energy liberated in the hydrolysis of ATP, Prof. G. E. Vladimirov ("On the Energetic Function of ATP in Cells") concluded that only 40-50% of the energy of respiration and glycolysis in brain tissue is used for ATP synthesis and that the effective coefficient of transformation of ATP into work for this tissue is very small.

The share of oxidative phosphorylation in biosynthesis was discussed in a paper by Prof. V. S. Shapot, "Oxidative Phosphorylation and the Mechanism of Biosynthesis." A. A. Remizov in a paper "On the Mechanism of the Acylation Reaction" gave an interesting treatment of the electron mechanism of acylation and the nature of the macroenergetic bond, which caused lively discussion.

In the paper "On the Similarity of Oxidative and Carbohydrate Metabolism in Cultures of Normal and Cancerous Tissue" V. I. Agol, V. N. Gershanovich, and R. N. Étingof strove to show that in three tissue cultures which they selected (monkey-kidney cortex, human tumor, strain GEP-2, and monkey "heart', cells) the unusual combination of intense aerobic and anaerobic glycolysis with damage to the oxidation of succinate and p-phenylenediamine could not be considered characteristic only for tumor cells, but was a general property of active, proliferating tissue. Prof. P. A. Kometiani, P. K. Tkshelashvili, and T. A. Ovsyanko ("The Use of Phosphate Esters of Choline, Ethanolamine, and Serine in Lipid Synthesis in the Brain") showed that the three compounds named, labeled with phosphorus, were included at high speed in the phospholipids of the brain in vivo, but in isolated systems this inclusion did not occur.

The only paper dealing with methods of investigation of oxidative phosphorylation, especially in isolated mitochondria, was given by Corresponding Member AN SSSR S. E. Severin, "Determination and Conditions for Retaining the Connection between Respiration and Phosphorylation." The paper gave a large number of indications of methods for quantitative determination of phosphorylation and work with isolated mitochondria; it emphasized that anserine and carnosine prevent swelling of mitochondria when they "age" and assure the relation between respiration and phosphorylation in experiments in vitro.

Some papers were given on the destruction of oxidative phosphorylation under the influence of radiation. The papers of L. V. Mytareva ("Processes of Oxidative Phosphorylation in Radiation Sickness in Experimental Animals") and of R. I. Skvortsova and P. F. Minaev ("Change in the Process of Oxidative Phosphorylation in Nervous Tissue under the Influence of Radiation") established the inhibiting action of ionizing radiation on oxidative phosphorylation. A. N. Klimov and E. G. Zezerov in the paper, "On the Destruction of Oxidative Phosphorylation under the Action of Antibiotics and Some Other Agents", reported that oxidative phosphorylation was sensitive to some antibiotics: gramicidin C, streptomycin, penicillin in high concentrations, and some others. Respiration was more stable to the action of inhibitors, but phosphorylation, on the contrary, was easily suppressed and its inhibition was removed with difficulty by different activators of tissue respiration. In the paper of M. Yu. Gaisinskaya some characteristics of carbohydrate-phosphorus metabolism in the pancreas were discussed.

The Division of Biochemistry of the IÉM delivered a series of papers on the nature of the regulation of the activity of phosphorylation enzymes. Prof. S. A. Neifakh, beginning this group of papers, reported "On Control and Maximum Rate of Glycolysis", a very well-considered idea of the two rates of glycolysis and the dependence of the maximum rate on the activity of the enzyme which has the lowest efficiency in a given series of reactions. In the paper of Prof. V. S. Il'in ("On Some Principles in the Study of the Mechanism of Central Regulation of Enzymatic Processes") he presented extensive experimental material on central regulation of the glycokinase reaction, which is one of the "bottlenecks" in carbohydrate metabolism, and reported a valuable study of the mechanism of action of hormones on enzymatic reactions of this type. The paper of N. I. Belyaeva ("On the Share of

the Nervous System in Regulating the Activity of Muscle Glycokinase") concerned the activity of glycokinase extracts from normal and denervated rabbit muscle. The reports of V. S. Il'in and S. A. Neifakh found confirmation for the cells of the Ehrlich ascitic tumor, where the "bottleneck" in glucose fermentation is the hexo- and 6-fructokinase reaction; when glycogen is used, the rate of glycolysis is determined by the phosphorylase (paper of M. P. Mel'nikova, "On the Enzymatic Steps Which Determine the Rate of Glycolysis in Cells of the Ehrlich Ascitic Carcinoma"). In the same direction, there was an attempt to connect the high level of glycolysis in cancer cells with the intensive synthesis of hexokinase (T. B. Kazakova, "Formation of Hexokinase in Cells of the Ehrlich Ascitic Carcinoma"). It was shown that cancer tissue actually synthesizes hexokinase energetically and this enters the blood vessels of the host.

A series of papers which concerned the relation of phosphorylation and movement was opened by Prof. D. L. Ferdman ("Data on the Study of Phosphorus Compounds in Muscle") who reported on the study of adenylic compounds of muscle in work, rest, and dystrophy by the method of paper chromatography and isotope labeling. S. A. Burnasheva reported on the relation of oxidative phosphorylation to the movement of spermatozoa and the reactions of the adenylic system when respiration and glycolysis were eliminated ("Significance of the Phosphorylation Process in the Movement of Semen Cells"). In the paper of N. N. Yakovlev ("Anaerobic and Respiratory Phosphorylation in Muscular Activity, Their Relation and Regulation") the question was considered of the relation of oxidative phosphorylation to length and intensity of muscular work, training, and the central nervous system and hormonal regulation. The paper of E. A. Matlina ("Effect of the Serum of Myasthenia Patients on Formation of Phosphocreatine") leads to the idea that the factor which suppresses formation of phosphocreatine in minced muscle in vitro and which is contained in the serum of patients with myasthenia is a phosphorylation substrate of unknown nature which competes with creatine for ATP.

The conference also considered the problem of the biochemistry of the nervous system. In the paper of N. A. Verzhbinska ("Oxidative Phosphorylation of the Brain in the Evolution of Vertebrates") the idea was expressed of the existence in brain tissue, of animals low in the evolutionary scale, of oxidation systems of the "water" type where the combination of oxidation with phosphorylation was absent or very slight (as in fish where the phosphorylation coefficient is only 0.05); the phylogenetically younger systems of the "land" type were characterized by a very close coupling of phosphorylation and oxidation. These data, obtained from extensive phylogenetic material, agree with the conclusions made on the basis of entirely different facts by V. P. Skulachev, whose paper was mentioned earlier. E. K. Balashova, M. N. Maslova, A. N. Panyukov, and V. I. Rozengart ("The Effect of Stimulation and Inhibition of the Central Nervous System on the Rate of Introduction of P^{32} into Phosphoproteins of the Brain") considered that neither the convulsive action of corasole and phosphacol nor the inhibition caused by diethylaminobenzene affected the rate of uptake of P^{32} in phosphoproteins, in spite of the results of other authors, according to whom such an effect occurs. In the paper of N. P. Lisovskaya and N. B. Livanova ("Oxidative Phosphorylation and Phosphoprotein Metabolism") they emphasized the significance of oxidative phosphorylation and the specific role of ATP in the renewal of phosphoprotein P. Continuing the study begun by V. S. Shapot, K. G. Gromova ("The Content and Formation of Coenzyme A in Brain Tissue in Its Stimulation and in Partial Anemia") found that the content of coenzyme A in the brain falls by 26.8% in anemia, and with stimulation by camphor, by 14%; the speaker considered these changes to be secondary.

Approximately a fourth of all the papers were devoted to problems of pharmacology and pathological physiology. In the paper of Prof. Karasik ("Macroenergetic Metabolism and Pharmacological Effects") he considered the general aspects of the relations of pharmacological agents and oxidative phosphorylation and traced the problems which arise in such studies. E. V. Moreva and A. I. Podlesnaya ("Comparative Rate of Posthumous Splitting of Macroenergetic Compounds in Different Stages of Postnatal Development of Animals") traced the relation of rate of onset of rigor mortis and the content of macroenergetic compounds, in connection with growth. In the opinion of I. P. Lapin, damage to oxidative phosphorylation by 2, 4-dinitrophenol causes periodic changes in heart function and the occurrence of the so-called period of Luciani. P. S. Vasilenko and V. O. Osinskaya presented a paper "Study of Adenosine Triphosphoric Acid and Adenosinetriphosphatase Activity in the Adrenal Gland in Connection with the Formation and Excretion of Adrenaline", and Yu. N. Stroikov gave a paper "The Effect of Pharmacological Blocking of Sympathetic Nervous Innervation on Some Phases of Phosphorus-Carbohydrate Metabolism in Skeletal Muscle."

In the paper of Prof. P. N. Veselkin, "On Changes in Energy Metabolism in Febrile States" he considered the general aspects of the problem of the febrile reaction in connection with energy metabolism. The basic ideas of

this paper received confirmation in the experimental study of E. P. Zdrodovskaya ("Processes of Oxidative Phosphorylation in Damage to Heat Exchange"). In a comparison of dinitrophenyl hyperthermia with fever produced by purified and unpurified bacterial pyrogens, it was shown that in the first case there was an increase in heat production and a fall in the intensity of oxidative phosphorylation, that is, a typical "dissociated" action, and in the second case only a delay in heat emission with normal heat production and along with this an increase in the level of oxidative phosphorylation. The paper of M. S. Gaevskaya and E. A. Nosova was also connected with questions of pathology, "The Phosphorylation Process in Brain Tissue at the Time of Dying and Revival of the Organism at Different Body Temperatures," as were those of Z. A. Raiko, I. R. Petrov, T. E. Kudritskaya, and M. N. Pertseva, "The Effect of Artifical Hypothermia on Some Phases of Carbohydrate-Phosphorus and Nitrogen Metabolism in the Brain and Heart when Circulation of the Blood is Completely Stopped," and of L. I. Niselovskaya, "The Effect of Injecting Diphtheria Toxin on the Process of Oxidative Phosphorylation."

Papers were also presented by Active Member of the Academy of Medical Sciences, USSR, A. E. Braunshtein and Prof. I. I. Ivanov.

The participants in the conference were shown a small exhibit and a demonstration of methods of chromatography which are used in the laboratory of animal-cell biochemistry of the Institute of Biochemistry of the Academy of Sciences, USSR.

A. E. Braunshtein summarized the results of the conference in his concluding words.

There can be no doubt as to the positive value of the conference on oxidative phosphorylation, which "inventoried" all, or almost all, the studies on this problem which are going on in the Soviet Union and permitted a meeting of the experimenters who are working on parallel lines to discuss debatable and unclear problems. The great number of those who engaged in discussion of the papers (200 people) indicates the importance of this and the activity of the auditors. Perhaps more important was the keennness and liveliness of the discussions, which in the general opinion, gave the meeting an atmosphere of spontaneity and serious criticism. The Division of Biochemistry of the IÉM, the moving spirit of the conference, performed a great and necessary act.

As to the general evaluation of the study of oxidative phosphorylation in the laboratories of the Soviet Union, there may be different opinions. It seems to us that the conference showed that studies on oxidative phosphorylation are being carried out in very different directions, in general at a good experimental and theoretical level. However, at the same time, the conference revealed large gaps, which are perhaps related to the most acute and important questions. Thus, there is evidently insufficient study of the mechanism of oxidative phosphorylation, the localization of phosphorylation in different links of the respiratory chain, the nature of the action of the various poisons, and other important questions. Photosynthetic phosphorylation was entirely missing from the plan of the conference, apparently because this new problem has only begun to be developed. From all this we can draw effective conclusions. We cannot forget that in the early stages of the development of the study of oxidative phosphorylation Soviet biochemists made two great contributions to the problem: the discovery of oxidative phosphorylation at the cellular level by V. A. Éngel'gardt (1930-1932) and the discovery of phosphorylation in the respiratory chain by V. A. Belitser (1939). Therefore, it is quite natural to attempt to maintain the initiative in the development of this important branch of biochemistry.

The IÉM has gathered the work of the conference to publish it; we can only hope that this intention will be carried out, and the sooner, the better.

A. A. Baev

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